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AUTHOR – AUTEUR

Full Name of Author – Nom complet de l'auteur

John Hales Bowen

Date of Birth – Date de naissance

Jan 12, 1955

Canadian Citizen – Citoyen canadien

[X] Yes / Oui [ ] No / Non

Country of Birth – Lieu de naissance

Canada

Permanent Address – Résidence fixe

67 Greystone Dr
Spruce Grove, Alta
T0E 2C0

THESIS – THÈSE

Title of Thesis – Titre de la thèse

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University – Université

Univ. of Alta.

Name of Supervisor – Nom du directeur de thèse

Dr. J.S. Colter

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THE USE OF MONOCLONAL ANTIBODIES IN STUDIES OF THE CAPSID PROTEINS AND THE POLY(U) POLYMERASE OF MENGO VIRUS

by

JOHN H. BOWEN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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PERMANENT ADDRESS:

67 Greystone Dr.

Spruce Grove,

Alberta

DATED September 30 1985

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled THE USE OF MONOCLONAL ANTIBODIES IN STUDIES OF THE CAPSID PROTEINS AND THE POLY(U) POLYMERASE OF MENGO VIRUS submitted by JOHN H. BOWEN in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

John A. Colter
Supervisor

Diane E. Taylor

Verner Paetkau

Wm Paranchych

D.H. Scraba

Jean Lucas-Lenard

Date September 30, 1985

To Kathryn

ABSTRACT

Hybridomas which produce monoclonal antibodies to capsid polypeptides of Mengo virus were isolated following the fusion of spleen cells from mice immunized with the 13.4S product produced by dissociation of Mengo virus at pH 6.2 (Mak *et al.*, 1971, 1974) with cells of the stable myeloma line Sp2/0-Ag14. Three hybridoma lines, MCP-2, 5 and 6, were found by the technique of renaturing Western blotting to produce monoclonal antibodies specific for Mengo capsid polypeptides α, β and β, respectively.

Monoclonal antibodies produced by four of the six hybridoma lines isolated were shown to be capable of neutralizing viral infectivity. The results of sucrose density gradient sedimentation analyses of virus-antibody mixtures indicate that three (MCP-1, 4 and 6) of these monoclonal antibodies neutralize infectivity by causing extensive cross-linking (aggregation) of the virions, even at antibody:virion ratios as low as 10. The fourth neutralizing monoclonal antibody, MCP-5, appears to block attachment of the virus to the cellular receptor, without inducing aggregation of the virions. This is believed to be the first report of a monoclonal antibody with anti-polypeptide β specificity that neutralizes in this manner.

The innoculation of mice with extracts of Mengo virus-infected cells from which the capsid polypeptides had been removed, and the subsequent fusion of the immune splenocytes with Sp2/0-Ag14 cells, led to the isolation of a hybridoma, designated MNCP-20, which produces a monoclonal antibody specific for the virus-encoded polypeptide E. This monoclonal antibody, when bound to Affi-Gel 10, provided an efficient reagent with which to isolate the virus-specific polypeptide E in homogeneous form by immunoaffinity chromatography.

The results obtained using an *in vitro* poly(U) polymerase assay suggest that the isolated polypeptide E is the Mengo virus-specific elongation polymerase. As was described previously for the partially purified poliovirus poly(U) polymerase (Flanegan and Baltimore, 1977), purified polypeptide E requires a template, a primer and magnesium ions for polymerase activity. Construction of a temperature-activity profile using the *in vitro* poly(U) polymerase assay revealed that the isolated polypeptide E is thermolabile. Measurements of circular dichroism and tryptophan fluorescence demonstrated clearly that the heat-induced loss of enzyme activity of polypeptide E can be correlated with a change in its conformation. The observed stabilization of both the enzyme activity and the secondary structure of the poly(U) polymerase against heat-induced change by the formation of a complex with the F(ab) fragment of the anti-polypeptide E IgG provides additional evidence that the

thermolability of the polymerase activity (in the absence of the F(ab) fragment) is the direct result of a conformational change in the enzyme.

In order to provide a possible explanation for the RNA$^-$ phenotype exhibited by several ts mutants of Mengo virus, the thermostabilities (with respect to both enzyme activity and conformation) of the poly(U) polymerase specified by these mutants were compared with that of the *wt* enzyme. The data show clearly that the RNA$^-$ phenotype of four (ts 135, ts 506, ts 520 and ts 620) of the six ts mutants studied can be attributed directly to a temperature-sensitive lesion which affects the elongation function of their respective polymerases.

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LIST OF ABBREVIATIONS

BME medium	- basal minimum essential medium
BSA	- bovine serum albumin
CD	- circular dichroism
cpm	- counts per minute
DME medium	- Dulbecco's modified Eagle's medium
DTT	- dithiothreitol
ELISA	- enzyme-linked immunosorbant assay
EMC virus	- encephalomyocarditis virus
FCS	- fetal calf serum
FMD virus	- foot-and-mouth disease virus
g	- centrifugal force relative to gravity
HAT medium	- hypoxanthine - aminopterin - thymidine supplemented DME - 10% FCS medium
HT medium	- hypoxanthine - thymidine supplemented DME -10% FCS medium
IB	- immunoprecipitation buffer
IgG	- immunoglobulin G
i.p.	- intraperitoneal
K.I.U.	- Kallikrein international units
MCP	- Mengo capsid polypeptide
MEM	- Eagles minimum essential medium
ME virus	- Maus-Elberfeld virus
µCi	- microcurie
µg	- microgram

LIST OF ABBREVIATIONS (continued)

µl	- microlitre
µm	- micrometer
mM	- millimolar
MNCP	- Mengo non-capsid polypeptide
mRNA	- messenger RNA
MW	- molecular weight
NEC	- natural empty capsid
nm	- nanometer
NP-40	- nonidet P-40
PAGE	- polyacrylamide gel electrophoresis
PBS	- phosphate buffered saline
PFU	- plaque forming unit
pI	- isoelectric point
pmole	- picomole
PMSF	- phenylmethylsulfonyl fluoride
PyCP	- polyoma virus capsid polypeptide
RF-RNA	- replicative form RNA
RI-RNA	- replicative intermediate RNA
RNAase	- ribonuclease
rpm	- revolutions per minute
SDS	- sodium dodecyl sulfate
TCA	- trichloroacetic acid
ts	- temperature-sensitive
wt	- wild-type
VP	- viral polypeptide
VPg	- viral genome protein

CHAPTER I

General Introduction

Picornavirus Classification

The term picornavirus was adopted in 1963 by the International Enterovirus Study Group to describe a family of very small (pico) rna viruses. The term has been applied formally to mammalian viruses that are small (less than 300 Å in diameter), lack an envelope and contain a single-stranded RNA genome. Although there are several plant, bacterial and insect viruses that meet these criteria, they have not yet been assigned genera.

Picornaviridae have been further sub-divided into four genera as shown in Table 1. Individual genera are distinguished by differences in pH stability and buoyant density in cesium chloride gradients (Andrewes *et al.*, 1978; Newman *et al.*, 1973; Scraba and Colter, 1974).

Physical-Chemical Properties of the Virion

The picornavirion is composed of a molecule of single-stranded RNA (30% by weight) enclosed in a protein capsid (70%). Neither carbohydrate nor lipid has been detected in the virion (Burness *et al.*, 1973). Extensive physical and hydrodynamic studies have revealed that all picornaviruses share certain basic properties. The particle is isometric with a hydrated diameter of approximately 30nm (Rueckert, 1971; Fenner *et al.*, 1974; Scraba and Colter, 1974). The best available values indicate that the picornavirion has a

TABLE 1

Picornavirus Classification Scheme

Genus	Viruses	Distinguishing Characteristics
Enterovirus	Human polio (3 serotypes) Human coxsackie A (23 serotypes) Human coxsackie B (6 serotypes) Human echo (32 serotypes) Enteroviruses of various animals	Buoyant density (in CsCl)=1.33-1.34g/ml Virions stable pH 3-10
Cardiovirus	Encephalomyocarditis (EMC) Mengo Columbia-SK Mouse Elberfeld (ME) MM	All serologically related Buoyant density=1.33-1.34g/ml Unstable at pH 5-7 in presence of Cl^- or Br^-
Rhinovirus	Human rhinovirus (113 serotypes) Bovine rhinovirus (2 serotypes)	Buoyant density=1.38-1.42g/ml Unstable below pH 5-6
Aphthovirus	Foot-and-mouth disease FMD; 7 serotypes)	Buoyant density=1.43-1.45g/ml Unstable below pH 5-6

Modified from Matthews (1982). The various picornavirus subgroups also have distinctive pathological properties discussed in detail in Andrewes *et al.* (1978) Echo is an abbreviation for "enteric cytopathic human orphan."

sedimentation coefficient (S°_{20} of 150-160S, a diffusion coefficient (D°_{20})of $1.44\text{-}1.47\times10^{-7}$ cm^2/sec and a partial specific volume ($\bar{v}$) of 0.68-0.70 ml/g. One can calculate a particle weight of $8.3\text{-}8.5\times10^{6}$ daltons by substituting these values into the Svedberg equation (Scraba *et al.*, 1967).

The RNA Component

The picornavirus genome is composed of a single strand of infectious RNA (Colter *et al.*, 1957; Alexander *et al.*, 1958; Bachrach *et al.*, 1964; Dimmock, 1966). With the exception of some human rhinovirus genomes, essentially equimolar amounts of adenylate, cytidylate, guanylate and uridylate are present (Newman *et al.*, 1973). No unusual nucleotides have been detected.

In 1981, the complete sequence of poliovirus type 1 RNA was reported by Kitamura *et al.* (1981) and Racaniello and Baltimore (1981). To date the complete RNA sequence of at least one representative of each of the four picornavirus genera has been reported (Palmenberg *et al.*, 1984; Forss *et al.*, 1984; Stanway *et al.*, 1984). The sequence data reveals significant differences in genome length ranging from 7433 nucleotides in poliovirus to 8450 in FMDV. The genome is polyadenylated at the 3' terminus, as are most eukaryotic mRNAs (Brawerman, 1974), the average length of the poly(A) tract varying from 35 residues in cardioviruses to 100 in aphthoviruses (Ahlquist and Kaesberg, 1979; Rueckert, 1985). The poly(A) tract, required for infectivity of the isolated

viral RNA (Spector and Baltimore, 1974; Burness *et al.*, 1977) is genetically encoded (Dorsch-Hasler *et al.*, 1975; Kitamura and Wimmer, 1980; Kitamura *et al.*, 1981) although post-transcriptional addition may also occur (Spector and Baltimore, 1974).

The longer RNA genomes found in the cardio- and aphthoviruses also carry a polycytidylic acid tract located between the 5' terminus and the beginning of the protein coding region (Black *et al.*, 1979). In the cardiovirus genome the poly(C) tract (80-250 residues) starts approximately 150 bases from the 5' end (Chumakov and Agol, 1976; Perez-Bercoff and Gander, 1977) while in apthoviruses it begins approximately 400 bases from the end (Harris and Brown, 1976; Rowlands *et al.*, 1978). The functional significance of the poly(C) tract remains questionable, particularly since there is no evidence of a poly(C) tract in the genomic RNAs of entero- and rhinoviruses (Brown *et al.*, 1974).

The 5' termini of most eukaryotic and viral RNAs are capped by the structure m'G(5')ppp(5')Np, which facilitates binding of the mRNA to the ribosome in the formation of stable initiation complexes (Banerjee, 1980). The cap structure is not present in picornaviral RNAs (either virion-associated or mRNA) but the genomic (virion) RNAs of all picornaviruses are covalently linked at the 5' terminus to a small virus-encoded protein, denoted VPg (Kitamura *et*

al., 1980; Wimmer, 1982). In poliovirus, VPg has been shown to be linked to the 5' terminal uridylic acid of the RNA by means of a tyrosine-O^4-phosphodiester linkage (Ambros and Baltimore, 1978; Rothberg *et al.*, 1978). The length of VPg varies only slightly (22 to 24 amino acid residues) in different picornaviruses (Rueckert, 1985). The genome sequences of most picornaviruses predict one form of the protein, but the FMDV genome appears to contain three VPg genes in tandem (Forss and Schaller, 1982) thereby explaining the existence of the three forms of VPg previously observed in the viral RNA (King *et al.*, 1980).

All nascent RNA strands have VPg attached to the 5' end, suggesting that this protein may be involved in an early event of RNA synthesis (Flanegan *et al.*, 1977; Nomoto *et al.*, 1977). Viral polysomal mRNA lacks VPg, but whether its removal by a cellular "unlinking enzyme" (Ambros *et al.*, 1978) is mandatory for translation seems doubtful from *in vitro* translation studies (Folini *et al.*, 1980; Dorner *et al.*, 1981). The finding that polysomal mRNA is not encapsidated (Levintow, 1974) has led to speculation that this protein may also play a role in viral morphogenesis.

The Protein Component

The picornavirus protein capsid is composed of four major polypeptide species, designated 1A, 1B, 1C and 1D in L-4-3-4 nomenclature (Rueckert and Wimmer, 1984), with average molecular weights calculated from the deduced amino acid

sequences (Rueckert, 1985) of 7300, 29200, 26000 and 32500 respectively. Aphthovirus capsid polypeptides were not included in these average values since sequence analysis of the viral genome (Forss *et al.*, 1984) predicts that their molecular weights, 1A(7362), 1B(24410), 1C(23746) and 1D(23840), differ significantly from those of the other picornaviruses. This observation explains the difficulty in identifying FMDV capsid polypeptides on the basis of their relative mobilities in polyacrylamide gel electrophoresis (Strohmaier *et al.*, 1982). There are approximately 60 copies of each of the four major polypeptides per virion, and, in addition, picornaviruses contain traces of polypeptide 1AB (Scraba and Colter, 1974) which is normally cleaved to give polypeptides 1A and 1B during the final viral maturation step (Jacobson and Baltimore, 1968).

The amino acid compositions of the individual capsid polypeptides reveal characteristics common to all four (Scraba, 1979). Notable features include a low content of sulfur-containing residues (2-4 mole %), a high content of proline (6-8 mole %) and other non-α-helix-forming residues (valine, isoleucine, serine, threonine and glycine; 35-40 mole %) and a substantial number of apolar residues (50 mole %). Optical rotatory disposion and circular dichroism measurements of the Mengo capsid polypeptides *in situ* (Scraba *et al.*, 1967; Kay *et al.*, 1970) had previously indicated that they have a low (5-10%) α-helical content.

Morphology of the Virion

Examination of poliovirus crystals by X-ray diffraction led to the conclusion that the virion possesses icosahedral (5:3:2) symmetry, and that the capsid is composed of 60 structurally equivalent asymmetric units (Finch and Klug, 1959). Technological advances in the complete structure of small plant RNA viruses (Harrision *et al.*, 1978; Liljas *et al.*, 1982) have led to a renewed interest in crystallographic studies of picornaviruses (Hogle, 1982; Erickson *et al.*, 1983; Boege *et al.*, 1984) in anticipation that the precise arrangement of the asymmetric subunits in the virions can be defined. Preliminary results show that poliovirus and rhinovirus crystals can be roughly isomorphous (Erickson *et al.*, 1983), and comparison of the X-ray diffraction patterns obtained from rhinovirus and Mengo virus crystals also reveal significant structural homologies (Luo *et al.*, 1984).

Much of the current information on the capsid architecture has been obtained from studies of the products formed by the acid dissociation of cardioviruses. In the presence of 0.1M chloride or bromide ions at pH 5 to 6.5 cardiovirions can be dissociated into infectious RNA, polypeptide δ, and a 13.4S subunit composed of equimolar amounts of polypeptides α, β and γ (Rueckert *et al.*, 1969; Mak *et al.*, 1970; Dunker and Rueckert, 1971; McGregor *et al.*, 1975). Disruption of hydrogen bonds and hydrophobic

interactions by 2M urea further dissociates the 13.4S subunits into 4.7S components with the same polypeptide composition. Molecular weight determinations and the polypeptide stoichiometry suggested that the 4.7S component is a monomer, ie. $(\alpha\beta\gamma)$, whereas the 13.4S subunit is a pentamer, ie. $(\alpha\beta\gamma)_5$, of the 4.7S monomer. The capsid structure is thus made up of 60 protomers (4.7S) associated by hydrophobic interactions into pentamers (13.4S), one of which is centered at each of the 12 vertices of an icosahedron (Dunker and Rueckert, 1971; Mak *et al.*, 1974).

Some insight into the spatial arrangement and interactions between individual capsid polypeptides has been obtained from studies of 1) the chemical labeling of surface polypeptides (Carthew and Martin, 1974; Lonberg-Holm and Butterworth, 1976; Lund *et al.*, 1977; Wetz and Habermehl, 1979), 2) the reaction of virions with monospecific antibodies (Lund *et al.*, 1977; Dernick *et al.*, 1983), 3) the treatment of virions with reagents that cross-link capsid proteins (Hordern *et al.*, 1978; Wetz and Habermehl 1979) and 4) cross-linking induced by exposure of the virions to ultraviolet light (Miller and Plagemann, 1974; Wetz and Habermehl, 1982). These studies have established two features of the architecture of the capsid. 1) polypeptide α is the most accessible and immuno-dominant protein and 2) polypeptide is inaccessible from the exterior of the virion, but can be cross-linked to the viral RNA on the interior (Putnak and Phillips, 1981a). Chemical cross-linking studies

by Hordern *et al.* (1978) suggest that polypeptides α, β and γ occupy relatively discrete domains within the protomer and that they are held together by α-γ-β or γ-α-β non-covalent interactions. The results also indicate that adjacent pentamers are held together by acid-sensitive α-β interactions, and that protomers are associated into pentamers through urea-sensitive α-α interactions.

Viral Replication

Attachment, Penetration and Uncoating

The initial event in the replication cycle of the virion is attachment to specific receptors located on the surface of susceptible cells. The number of saturable cellular receptors has been estimated to range from 10^4 to 10^5 per cell (Lonberg-Holm, 1981). Picornaviruses do not all share common receptors, as evidenced by the results of attachment-competition experiments (Crowell and Landau, 1983; Abraham and Colonno, 1984), and by differences among the receptor families with respect to susceptibility to inactivation by proteolytic enzymes and neuraminidase, and to blocking by monoclonal antibodies (Campbell and Cords, 1983; Minor *et al.*, 1984). Cardioviruses are relatively unique among picornaviruses in their requirement for sialic acid-containing receptors for attachment (Burness, 1981). Inhibition studies with concanavalin A indicate that the cellular receptors for polioviruses and Group B coxsackieviruses are glycoproteins containing α-D-mannosyl-like residues (Lonberg-Holm, 1975; Krah and

Crowell, 1985).

The identification of the viral polypeptide(s) involved in the attachment of the virion to the cellular receptor remains the subject of much controversy. Some of the confusion has arisen because of the perhaps naive tendancy of investigators to equate neutralization with the blocking of viral attachment (for a review see Crowell and Landau, 1983). Lonberg-Holm and Philipson (1980) have proposed that the viral 'antireceptor' site is composed of several polypeptides acting cooperatively to provide a unique conformation for receptor recognition. The best evidence to date using monospecific sera (Lund *et al.*, 1977) and monoclonal antibodies (Emini *et al.*, 1983a; Baxt *et al.*, 1984) indicates that polypeptide α is involved in attachment to the cell receptor.

Viral attachment to the cellular receptor is primarily an electrostatic interaction (Crowell and Landau, 1983). The initial attachment of the virion is reversible, but this 'loose' attachment of the virion is rapidly converted to an irreversible virus-receptor complex (Lonberg-Holm and Philipson, 1974). The transition to a tightly bound virus-receptor complex is temperature dependent in enteroviruses and rhinoviruses (Lonberg-Holm and Korant, 1972; Lonberg-Holm and Whitely, 1976; Crowell and Siak, 1978) suggesting that membrane fluidity and multivalent binding to receptor units may play an important role in the

attachment of these viruses. Receptors for cardioviruses and aphthoviruses, however, may be clustered on the cell surface, resulting in an attachment process that is virtually temperature-independent (Mak *et al.*,1970; Levanon *et al.*, 1977; McClintock *et al.*, 1980; Baxt and Bachrach, 1980).

Events which follow receptor-binding are less well established and are often difficult to interpret. In susceptible cells, there is a rapid loss in the ability to recover infectious virus, a process called eclipse, accompanied or followed by uncoating of the viral RNA. Unfortunately, only a very small proportion of the attached virions (approximately 1%) is productively uncoated (Mandel, 1965). With entero- and rhinoviruses a large proportion of the virus population (50 to 80%) is eluted as non-infectious A particles (Crowell and Landau, 1983); A particles being characterized by their slower sedimentatio rate in sucrose gradients (90% of that of infectious virions), loss of polypeptide 1A, resistance to RNAse, sensitivity to proteases and their inability to reattach to cells (Joklik and Darnell, 1961; Fenwick and Cooper, 1962; Crowell and Philipson, 1971; Cords *et al.*, 1975). There is some evidence that with poliovirus and coxsackievirus B3 there is a further sequential loss of polypeptide 1B leading to the formation of so-called B and/or C particles (Lonberg-Holm and Philipson, 1974; DeSena and Mandel, 1976, 1977; Guttman and Baltimore, 1977; McGeady and Crowell, 1981). A particle

formation has not been detected in studies with either aphtho- or cardioviruses, possibly due to the inherent lability of the particles (Brown *et al.*, 1962; Hall and Rueckert, 1971; Baxt and Bachrach, 1980, McClintock *et al.*, 1980).

Some doubt has been cast on the hypothesis that A particle formation results from abortive penetration and uncoating of the virus at the cell membrane (Crowell and Landau, 1983) by recent electron microscopic evidence which indicated that penetration by polio and ME viruses occurs through receptor-mediated endocytosis (Zeichhardt *et al.*, 1985). The formation of A particles may, however, provide a model for the uncoating process. Reagents such as rhodanine for echovirus 12 (Eggers, 1977), trimethylpyrimidine (Lonberg-Holm *et al.*, 1975) and arildone (McSharry *et al.*, 1979; Caliguiri *et al.*, 1980; Schrom *et al.*, 1982) for poliovirus, and Ro 09-0410 (Ninomiya *et al.*, 1984) for rhinovirus, not only block viral replication, but stablize the virus against thermal and alkaline degradation which normally results in the formation of products similar to A particles. These reagents appear to block viral replication by the selective inhibition of the uncoating process.

The entry of several viruses into cells is regulated by receptor-mediated endocytosis via coated pits (Helenius *et al.*, 1980, 1982; White *et al.*, 1981; Matlin *et al.*, 1982; Sato *et al.*, 1983). Endocytosed picornavirus particles can

be entrapped in endosomes and/or lysosomes by raising the pH of intracellular vesicles with weak bases (Zeichhardt *et al.*, 1985). Results from several studies using a variety of weak bases and ionophores suggest that the entry of the viral genome into the cytoplasm occurs through acidic vesicles in the cases of poliovirus type 1, human rhinovirus 2, FMDV and ME virus, and through neutral or slightly alkaline vesicles in the case of EMC virus (Carillo *et al.*, 1984; Madshus *et al.*, 1984a, 1984b; Zeichhardt *et al.*, 1985). The exact mechanism by which the viral genome is delivered from the intracellular vesicle into the cytoplasm remains unclear. Poliovirus, though hydrophilic at neutral pH, exposes hydrophobic regions at low pH, and it has been suggested that intercalation of these exposed hydrophobic regions into the vesicular membrane results in the release of the viral RNA into the cytoplasm (Lonberg-Holm and Whitely, 1976; Madshus *et al.*, 1984a).

Alterations of Cellular Metabolism

Infection by picornaviruses causes a rapid inhibition of host-cell macromolecular synthesis (Lucas-Lenard, 1979). The rate and extent of inhibition depends on the strain of virus, the multiplicity of infection and the cell type involved (McCormack and Penman, 1967; Jen *et al.*, 1980; Otto and Lucas-Lenard, 1980).

The rapid inhibition of protein synthesis in HeLa cells following infection with poliovirus is accompanied by the

inactivation of cap-binding factors essential for the initiation of translation of host mRNAs but not required for translation of viral RNA (Helentjaris and Ehrenfeld, 1978; Rose *et al.*, 1978). Recent studies with poliovirus and rhinovirus 14 suggest that this inhibition is caused by cleavage of the 220,000 dalton (p220) subunit of the cellular "cap-binding protein complex" (also known as CBP complex, CBP II and eIF-4F) required for the attachment of capped mRNAs to ribosomes (Tahara *et al.*, 1981; Etchison *et al.*, 1982, 1984; Grifo *et al.*, 1983; Edery *et al.*, 1983; Lee *et al.*, 1985; Etchison and Fout, 1985). Poliovirus-specific polypeptides 2C and 3C (viral protease) are not directly responsible for the cleavage of the p220 subunit, and it has been proposed that cleavage may be due either to a hitherto unidentified viral protease or to an induced cellular activity (Lloyd *et al.*, 1985; Lee *et al.*, 1985).

Cardioviruses inhibit host cell translation by a mechanism distinctly different from that observed with poliovirus (Lucas-Lenard, 1979; Jen *et al.*, 1980; Mosenkis *et al.*, 1985). With EMC and Mengo virus, competition between the viral RNA and host mRNAs for initiation factors may play an important role in the shutoff phenomenon (Jen *et al.*, 1980; Perez-Bercoff and Kaempfer, 1982; Rosen *et al.*, 1982). It has been suggested that the inhibition of host-cell protein synthesis by EMC virus may be the result of virus-induced changes in membrane permeability to monovalent cations (Carasco and Smith, 1976; Lacal and Carasco, 1982), but it

would appear that these changes occur too late in the replication cycle to be responsible for the observed inhibition (Nair, 1984).

It has been shown that, in L cells, the rapid inhibition of protein synthesis by Mengo virus is accompanied by a disaggregation of polysomes and a resultant increase in 80S ribosomes (Colby *et al.*, 1974; Egberts *et al.*, 1976). Recently, Pensiero and Lucas-Lenard (1985) have reported the presence in Mengo-infected L cells of an inhibitor which binds to ribosomes, causing an accumulation of 80S initiation complexes that cannot engage in elongation. The authors propose a model in which either a virus-specific or a viral-induced cellular protein reversibly inactivates 80S initiation complexes, and in which, under the resulting conditions of limited protein synthesis, the Mengo mRNA competes successfully with host-cell mRNAs for the remaining functional initiation factors.

Synthesis of cellular ribosomal and mRNA also declines soon after infection with picornaviruses (Baltimore, 1969). Inhibitors of protein synthesis have been found to prevent this inhibition of cellular RNA synthesis, thus implicating a viral protein in the shutoff process (Baltimore *et al.*, 1963). Measurements of alpha-amanitin-sensitive transcription in isolated nuclei indicated that in virus-infected cells inhibition of RNA polymerase II may preceed inhibition of polymerase I and III (Schwartz *et al.*, 1974; Apriletti

and Penhoet, 1974). However, when freed from their nucleoprotein complexes all three polymerases were found to be fully active. Crawford *et al.* (1981) have suggested that the putative viral-coded inhibitor may act on an unidentified factor required by polymerase II.

The inhibition of DNA synthesis during infection with picornaviruses appears to be a result of inhibition of host-cell protein synthesis. The effect can be mimicked in uninfected cells by antibiotic inhibitors of protein synthesis (Hand *et al.*, 1971; Hand and Tamm, 1972).

Polypeptide Synthesis and Cleavage

As mentioned previously, the complete nucleotide sequence of picornaviral RNA has been determined for at least one representative of each of the 4 genera (Kitamura *et al.*, 1981; Palmenberg *et al.*,1984; Forss *et al.*, 1984; Stanway *et al.*, 1984). The RNAs are characterized by a long open reading frame (6500-7000 nucleotides) which codes for a single polyprotein translation product. Under normal conditions the polyprotein (MW ~ 250,000) cannot be detected, since it undergoes primary cleavages as it is being synthesized to yield the precursors of all the viral capsid and nonstructural polypeptides (for a review see Lucas-Lenard, 1979).

The cleavage pattern for the generation of Mengo virus polypeptides is shown in Figure 1. The gene order was determined mainly by the technique of pactamycin mapping (Paucha

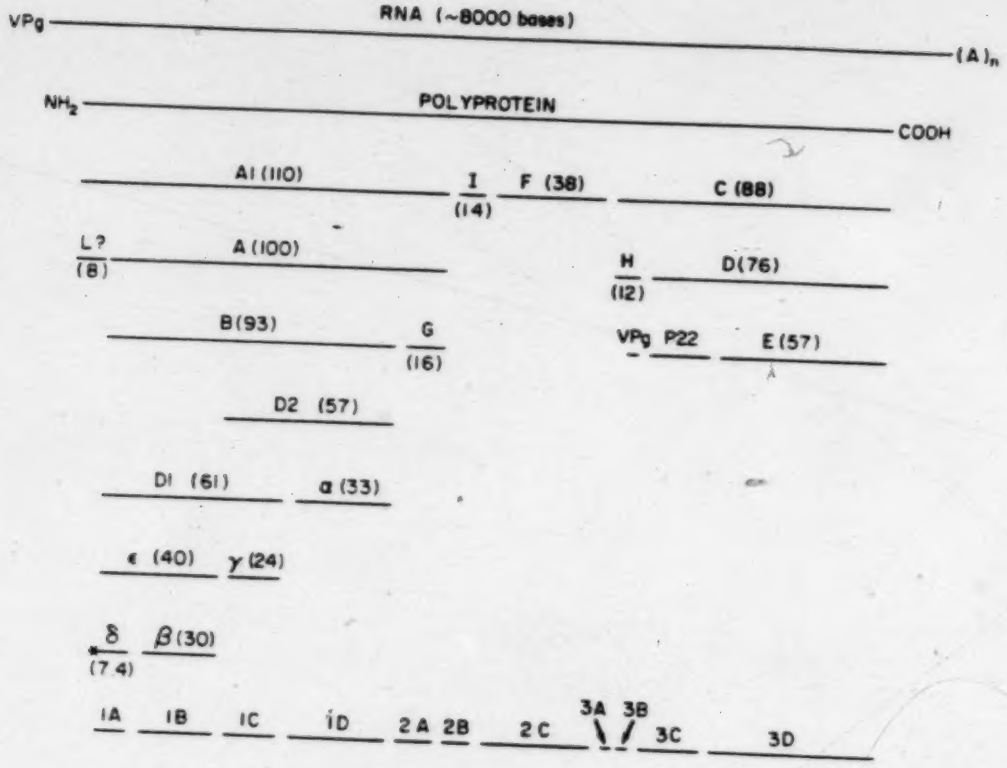

Figure 1. Cleavage map of Mengo virus-specific polypeptides. The molecular weights (in thousands) shown in brackets were determined from SDS-PAGE. The standard picornaviral 4-3-4 nomenclature (Rueckert and Wimmer, 1984) is given at the bottom of the figure.

et al., 1974); however assignments of the location of polypeptide L, I, H, VPg and p22 were made by analogy to EMC virus (Palmenberg *et al.*, 1984). The primary cleavages of the nascent polyprotein to give polypeptides A1, I and F, and C are thought to be catalyzed by a cellular protease (Korant *et al.*, 1980). The leader polypeptide (L) found only in cardioviruses and aphthoviruses has not yet been demonstrated in the Mengo virus system, but in the case of EMC virus (Palmenberg *et al.*, 1984) it is cleaved from the amino terminus of A1 to give A by what may be an autocatalytic process (Stanway *et al.*, 1984). Subsequent secondary cleavages are mediated by a viral protease. The viral-encoded protease has not yet been isolated in pure form, but antibody inhibition studies (Hanecak *et al.*, 1982) and its expression from cloned cDNA fragments (Hanecak *et al.*, 1984; Klump *et al.*, 1984) have confirmed its earlier identification from cell-free translation studies (Pelham, 1978; Gorbalenya *et al.*, 1979; Palmenberg *et al.*, 1979; Shih *et al.*, 1978) as p22 or 3C. It has been suggested that the viral protease is generated by intramolecular self-cleavage of polypeptide D (Rueckert *et al.*, 1980; Palmenberg and Rueckert, 1982; Hanecak *et al.*, 1984; Klump *et al.*, 1984). The poliovirus protease appears to have absolute specificity for the gln-gly linkage (Kitamura *et al.*, 1981), whereas in cardioviruses, peptide bond cleavages attributed to p22 include both gln-gly and gln-ser (Ziola and Scraba, 1976; Palmenberg *et al.*, 1984). The FMD virus protease possesses

an even broader range of specificity, cleaving glu-ser, glu-gly and glu-thr bonds (Forss *et al.*, 1984). Inhibition of the EMC protease with iodoacetamide and N-ethylmaleimide indicates that one or more sulfhydryl groups are required for its activity, i.e. that p22 is a cysteine protease (Pelham, 1978; Gorbalenya *et al.*, 1983).

The final 'morphogenetic' cleavage of ϵ to $\delta + \beta$ occurs during maturation of the progeny virions. The amino-terminus of polypeptide δ is blocked during post-translational processing (Ziola and Scraba, 1976). Polypeptide C, representing the 3' region of the genome contains the sequences of VPg, the protein linked to the 5' terminus of the viral genome, the viral protease p22, and the polymerase, polypeptide E (Lund and Scraba, 1979).

Replication of Viral RNA

Synthesis of viral RNA commences within 30 minutes of infection and proceeds at an exponential rate for the next 3 to 4 hours, at which time synthesis becomes linear (Baltimore, 1969). The synthesis of viral RNA is asymmetric in that only 5 to 10% of the total viral RNA isolated from infected cells consists of minus strands (Baltimore and Girard, 1966). The mechanism by which viral RNA synthesis is controlled has not been elucidated completely, but a still viable hypothesis is that the removal of plus RNA from replicative pools for translation or encapsidation is an important factor in the regulation of RNA synthesis

(Baltimore, 1969).

Transcription of picornaviral RNA occurs in a replication complex, consisting of the viral RNA and several virus-specific and host polypeptides in tight association with smooth cytoplasmic membranes (for a review see Perez-Bercoff, 1979). In addition to the newly synthesized viral RNA, two other structures containing viral RNA can also be isolated from infected cells. One such structure, the replicative intermediate (RI), has been shown by *in vitro* pulse-chase experiments (Girard, 1969; McDonnell and Levintow, 1970) to be the actual site of synthesis of virion RNA. The RI consists of a double-stranded RNA "core" with several (6 to 8) nascent RNA chains of positive sense and of variable length, hydrogen-bonded over short regions to the template (Baltimore, 1969; Oberg and Philipson, 1971; Richards *et al.*, 1984). This suggests a semi-conservative mode of replication.

A second structure, the so-called replicative form (RF) RNA, a double-stranded molecule containing unit-length complementary plus and minus strands, is also found in infected cells. As it accumulates only toward the end of the infectious cycle (Baltimore and Girard, 1966), and bears no apparent precursor-product relationship with viral RNA (Baltimore, 1968; Girard, 1969), it is believed to be a by-product of the replication process.

The development of an *in vitro* poly(A).oligo(U)-dependent poly(U) polymerase assay by Flanegan and Baltimore (1977) has led to the identification and characterization of the poliovirus replicase, 3D (Flanegan and Baltimore, 1970; Baron and Baltimore, 1982c). The template-dependent viral polymerase requires an oligo(U) primer for the *in vitro* initiation of RNA synthesis, but is capable of elongation without additional factors (Flanegan and Van Dyke, 1979; Van Dyke *et al.*, 1982; Tuschall *et al.*, 1982). The *in vitro* system however, is considered to be a model for the synthesis of minus strands only, since the main product of the *in vitro* reaction is double-stranded RNA (Dasgupta *et al.*, 1980; Baron and Baltimore, 1982b). Several investigators have recently isolated a series of VPg-precursors from cells infected with poliovirus (containing portions of the P2 region of the genome) and have suggested that these precursors may donate a VPgpUpU "primer" for the elongation of nascent minus strands (Nomoto *et al.*, 1977; Baron and Baltimore, 1982d; Morrow and Dasgupta, 1983; Crawford and Baltimore, 1983; Takegami *et al.*, 1983a,b). However, the direct participation of VPg, or any of its precursors, in the process of initation has not been demonstrated unequivocally.

A cytoplasmic protein (MW 67,000) found in uninfected HeLa cells and called 'host-factor' is capable of restoring replicase activity to the purified viral polymerase in the absence of an oligo(U) primer (Dasgupta *et al.*, 1980; Baron

and Baltimore, 1982a; Dasgupta, 1983). The mechanism by which this host factor stimulates the initiation of viral replication is also the subject of much speculation. Recent evidence indicates that 'host-factor' may be a protein kinase, which, in addition to stimulating poliovirus RNA replication, phosphorylates not only itself but also the α-subunit of eukaryotic protein synthesis initiation factor eIF-2 (Morrow *et al.*, 1985).

A protein having terminal uridylyl transferase activity (MW 60,000) isolated from rabbit reticulocytes by Andrews *et al.* (1985) can replace the Hela cell host factor in the replicase assay. The authors propose a model in which a terminal uridylyl transferase adds a short oligo(U) tail to the 3'-terminal poly A of the genomic RNA, which can then snap back to form a template/primer for the viral polymerase. This model is consistent with the isolation from both *in vivo* (Senkevich *et al.*, 1980) and *in vitro* (Young *et al.*, 1985) systems of nascent minus strand RNA covalently-linked to a plus strand template. The subsequent separation of the double-stranded product would require a specific nuclease or nicking enzyme (VPg-precursor ?). Unfortunately, the model does not take into account the observation that the HeLa cell host factor does not stimulate the poly(U) polymerase activity of the purified viral polymerase (Dasgupta *et al.*, 1980) *in vitro*.

The mechanism of initiation and elongation of plus strands from minus strand templates remains unknown. However, in the absence of a 3'-terminal poly(A) tail, it is believed that at least the process of initiation should be different from that described for the synthesis of minus strands.

Virion Assembly

After two decades of fairly intensive study, the precise mechanism of picornaviral morphogenesis remains unresolved. The isolation and identification of a series of "subviral" particles, has in itself generated more questions than answers (for a review see Putnak and Phillips, 1981a). There is a general agreement, however, that a 14S capsid precursor, with polypeptide composition $(\epsilon\alpha\gamma)_s$, occupies a central role in virion assembly (Watanabe *et al.*, 1962; Phillips *et al.*, 1968; McGregor *et al.*, 1975; McGregor and Rueckert, 1977). It is with respect to the nature of the ensuing steps that considerable divergence of opinion has arisen.

Except in the case of cardioviruses (McGregor *et al.*, 1975) natural empty capsids (NEC), which sediment at ~80S, can be isolated from cells infected with picornaviruses (Cowan and Graves, 1966; Maizel *et al.*, 1967; Korant *et al.*, 1975). Natural empty capsids are N antigenic (possess the "native" conformation expressed in infectious virus) and are reversibly dissociable into 14S subunits (Marongiu *et al.*,

1981). *In vitro* studies of the assembly of poliovirus 14S subunits into NEC suggest that the process requires a virus-specified or induced morphopoietic factor which is present in extracts of infected cells (Putnak and Philips, 1981b, 1982; Rombaut *et al.*, 1984).

Originally it was thought that the virion RNA was inserted into NEC, and that this was followed by the maturation cleavage of 1AB→1A+1B (Jacobson and Baltimore, 1968; Fernandez-Thomas and Baltimore, 1973; Yafal and Palma, 1979). However, the hypothesis that NEC are intermediates in virion assembly has been questioned seriously. Stable empty capsids have never been demonstrated in cardiovirus systems (Putnak and Philips, 1981a), nor has a precursor-product relationship between NEC and virions been established in cells infected with either FMDV (Rowlands *et al.*, 1975) or bovine enterovirus 1 (Su and Taylor, 1976). Furthermore, in some cell types (e.g. MiO cells) infection with poliovirus does not result in the formation of NEC (Ghendon *et al.*, 1972). Thus, it has been suggested that empty capsids are merely a storage form of 14S subunits or, in some cases, an artefact of the isolation procedure (Marongiu *et al.*, 1981).

With respect to cardioviruses, Lee and Colter (1979) isolated a 53S subviral particle from Mengo virus-infected cells. Depending on the ionic strength of the buffer, the 53S particle was found to either dissociate into 14S subunits (< 0.08M KCl) or dimerize into 75S shells (0.15M KCl).

It is again difficult to assess whether the 53S particle represents a true intermediate in virion assembly or an aggregate in equilibrium with the 14S capsid precursor. It remains possible that poliovirus natural empty capsids, with a polypeptide composition similar to that described for the Mengo virus 75S particle, lack the full complement of 14S subunits found in native virions, thus leaving a hole through which the virion RNA might be inserted (Lee and Colter, 1979).

Perhaps the key to the puzzle of assembly lies in the packaging of the viral RNA into a condensed ball small enough to fit into the protein capsid (Rueckert, 1985). Available evidence from short pulse experiments with radioactive uridine suggests that assembly occurs in tandem with replication of the viral RNA on the smooth cytoplasmic membranes (Caliguiri and Mosser, 1971; Yin, 1977). Whether the 14S subviral particles coalesce around the nascent viral RNA, or whether the RNA is inserted into some form of pre-formed shell, has not yet been determined.

Viral Antigenic Determinants and Neutralization

The identification of picornaviral antigenic determinants has been greatly faciliated by the simultaneous development of monoclonal antibody and synthetic peptide technologies (for a review see Brown, 1983). By the application of these techniques, intact poliovirions have been found to possess a major antigenic determinant located 93 to

100 amino acids from the N-terminus of polypeptide 1D, (VP1), as well as a minor site near the C-terminus of the same polypeptide (Minor *et al.*, 1983, 1985). A similar set of neutralizing antigenic determinants has been identified on polypeptide 1D (between residues 145-179 and 201-213) of FMDV (Strohmaier *et al.*, 1982; Bittle *et al.*, 1982; Pfaff *et al.*, 1982; Robertson *et al.*, 1984; Weddell *et al.*, 1985). However, antibodies directed against these antigenic determinants may not be the only ones involved in neutralizing viral infectivity, since the isolated poliovirus polypeptides 1B, 1C, and 1D (VP2, VP3 and VP1) are all capable of eliciting a neutralizing antibody response. In contrast to the finding with poliovirus and FMDV, Sherry and Rueckert (1985) have obtained evidence for the existence of at least two dominant neutralizing antigenic determinants on human rhinovirus 14, located on two separate polypeptides (1C and 1D).

To date, the emphasis of these investigations has been on the identification of neutralizing antigenic determinants, and on the development of novel vaccines for picornaviruses of economic importance, rather than on the mechanism(s) of neutralization. However, several mechanisms for viral neutralization, based on knowledge gained from the use of either polyclonal antisera or monoclonal antibodies, have been proposed (for a review see Dimmock, 1984). From the results of studies of the antibody-mediated shift in the isoelectric point of poliovirus, Mandel (1976) postulated

that neutralizing antibodies induce a conformational change in the viral capsid which prevents the subsequent uncoating of the virion. This particular phenomenon appears to require bivalent attachment of the antibody to the virion (Emini *et al.*, 1983a,d). However, a recent study by Brioen *et al.* (1985) failed to find any quantitative correlation between neutralization and the antibody-mediated shift in the pI of poliovirus.

While each virion contains 60 potential binding sites for a particular IgG, it has been shown that the binding of as few as 4 molecules of a monoclonal IgG per virion is capable of neutralizing viral infectivity. Based on this observation, Icenogle *et al.* (1983) have proposed that cross-linking of the virus pentameric subunits, $(\alpha\beta\gamma)_5$, by neutralizing antibodies prevents virion uncoating. A significant reduction in viral infectivity can also be achieved by antibody-mediated aggregation of the virus (Icenogle *et al.*, 1983; Brioen *et al.*, 1983; Baxt *et al.*, 1984; Thomas *et al.*, 1985). A final, apparently less common mechanism of neutralization involves the specific blocking of viral attachment to its cellular receptor (Emini *et al.*, 1983a; Baxt *et al.*, 1984).

Monoclonal Antibodies

Monoclonal antibody technology was developed by Kohler and Milstein (1975) during their studies on antibody diversity. It would be difficult to over-emphasize the

importance of this development, for which these investigators were awarded the Nobel prize in Medicine in 1984. The technique involves the fusion of immune B lymphocytes with cells of stable myeloma lines of the same species to yield established hybridoma lines, each of which produces an antibody with a single specificity (i.e. directed against a single antigenic determinant). The specific applications of monoclonal antibody technology are too numerous to document here. Suffice it to say that this technology has revolutionized the fields of clinical diagnostics, therapeutics and imaging, and has enjoyed widespread use in basic research including the techniques of immunopurification, epitope characterization and genetic analysis (for reviews see Kohler, 1985; Milstein, 1985; Newmark, 1985).

In this chapter alone, the work of several investigators who have made use of monoclonal antibodies in studies of picornaviral neutralization has been cited. Monoclonal antibodies by their very nature have made it possible to identify viral antigenic determinants (antibody binding sites), and this in turn has led to the evolution of a better understanding of the process of virus neutralization.

In this thesis, the application of monoclonal antibody technology to two separate lines of investigation of the Mengo virus system are described. They are: 1) studies of

neutralizing and non-neutralizing antigenic determinants present in the 13.4S subviral particle produced by pH-inactivation (dissociation) of Mengo virus (Mak *et al.*,1971, 1974); and 2) studies of the *in vitro* enzyme activities and conformational stabilities of preparations of Mengo virus-specific RNA polymerase (polypeptide E) specified by *wt* Mengo virus and by several RNA$^-$ ts mutants of Mengo virus, which were purified to homogeneity by immunoaffinity chromatography using an anti-polypeptide E monoclonal IgG.

CHAPTER II

Routine Materials and Methods

Media

Tissue Culture Media

Eagle's basal medium (BME) for L-cell monolayer cultures, Eagle's minimum essential medium (MEM) for L-cell suspension cultures and Dulbecco's modification of Eagle's medium (DME) for myeloma and hybridoma cultures were obtained in powder form from Flow Laboratories, McLean, Va. After dissolving the powder in distilled, deionized water, sodium bicarbonate was added to a final concentration of 0.168% (BME), 0.20% (MEM) or 0.37% (DME). All media were sterilized by Millipore filtration (0.22 μm pore size - Millipore Corp., Bedford, MA.), and before use were supplemented with penicillin G (Glaxo Laboratories, Toronto, Ont.) and streptomycin sulfate (Sigma Chemical Co., St. Louis, MO.) to final concentrations of 100 I.U./ml and 50 μg/ml respectively. Serum requirements for the individual cell lines were met by the addition of fetal calf serum (FCS, Grand Island Biological Company, Grand Island, N.Y.) to final concentrations ranging from 1% to 10%, depending on the cell line and the experimental protocol involved.

Amino Acid Deficient Medium

This medium was similar in composition to Eagle's basal medium except that it contained no amino acids other than glutamine. After addition of sodium bicarbonate to a final

concentration of 0.06%, the medium was sterilized by filtration and supplemented with 1% FCS and antibiotic as described above.

Virus Diluent

The phosphate-buffered saline (PBS) described by Dulbecco and Vogt (1954) was supplemented with 0.2% bovine serum albumin, fraction V (Sigma), 0.02% phenol red (Fisher Scientific Co., Fairlawn, N.J.) and antibiotics as described above.

Overlay Diluent

This medium contained three times the normal concentration of Hank's salts, six times the normal concentrations of both basal Eagle's amino acids and vitamins (Flow Laboratories), 0.78% sodium bicarbonate, five times the usual concentrations of penicillin and streptomycin and 30% inactivated (56° for 1 hour) calf serum (Flow Laboratories).

Agar Overlay

Agar overlay was prepared by mixing one volume of overlay diluent and two volumes of a 1.6% solution of Noble agar (Difco Laboratories, Detroit, Mich.) in distilled water.

Cultured Cells

L Cells

Eagle's L-929 strain of mouse fibroblasts (Sanford *et. al.*, 1948) were used for both the propagation and plaque

assay of Mengo virus. They were obtained from the American Type Culture Collection, Rockland, MD., and were passaged in 1-litre Blake bottles (Kimble Products, Owens Illinois Co., Toledo, OH.) at 37°. Upon reaching confluence, cells were harvested by a brief incubation with trypsin (0.25%, Difco) in a buffer containing 10mM sodium phosphate pH 7.4, 142.8mM sodium chloride and 2.8mM potassium chloride. Cells from one bottle were resuspended in fresh BME-5% FCS and used to maintain the Blake bottle stock. The remaining cells were resuspended in MEM-5% FCS, transferred to 2-litre spinner flasks (Bellco Biological Glassware, Vineland, NJ) at a concentration of $5x10^5$ cells/ml and grown at 37°. L cells were also grown as monolayers in roller bottles (490mm x 110mm diameter - Bellco Biological Glassware) pre-coated with FCS in order to facilitate the attachment of cells. The roller bottles were seeded with 10^8 cells harvested from suspension culture and resuspended in 100ml BME-5% FCS. The bottles were rotated on a Bellco roller apparatus at approximately 0.4 rpm.

Myeloma Sp2/0-Ag14

The mouse myeloma cell line, Sp2/0-Ag14, used in the production of hybridomas was originally isolated by Shulman *et al.* (1978). This particular myeloma cell synthesizes no immunoglobulin chains, is deficient in hypoxanthine-guanine phosphoribosyl transferase and is resistant to 8-azaguanine (20μg/ml). The cells were obtained from Dr. P.W.K. Lee, Department of Microbiology and Infectious Diseases,

University of Calgary. Cells of this line were maintained in suspension culture (3-8x10^5 cells/ml) in 75cm^2 tissue culture flasks (Lux Plasticware) in DME-10% FCS, supplemented with 0.1mM 8-azaguanine (Sigma) to prevent the generation of revertants.

Virus

The plaque variant of Mengo virus originally isolated and designated as M-Mengo by Ellem and Colter (1961) was used throughout these studies.

Virus Growth in Roller Bottles

Confluent monolayers were infected at an approximate multiplicity of 2 PFU/cell with Mengo virus suspended in 20ml BME-1% FCS. The bottles were rotated at 0.4 rpm for 18 to 20 hours by which time the majority of the cells had lysed and could be dislodged easily from the glass by shaking.

Virus Purification

Virus was purified by the method described by Ziola and Scraba (1974), except that the final concentration of methanol employed in the initial precipitation step was 30%, and the suspension was allowed to stand overnight at -20° before the virus and cellular macromolecules were collected by low speed (5000xg) centrifugation. Subsequent treatment with α-chymotrypsin, differential centrifugation, sedimentation through a 30% sucrose solution, and equilibrium

centrifugation in Cs_2SO_4 were performed as described by these investigators.

Preparation of Radioactively-Labeled Virus

Confluent monolayers of L cells in 150cm² tissue culture flasks (Falcon Plastics, Fisher Scientific Co.) were infected with Mengo virus at a multiplicity of 20 PFU/cell in BME-1% FCS. Three hours after infection, [5-³H] uridine (Amersham, Oakville, Ont.) was added to a final concentration of 50μCi/ml. Cultures were harvested when cytopathology was extensive (18-20 hr post-infection), and radiolabeled virus was purified by the procedure outlined in the preceding section.

Plaque Assay of Infectious Virus

Virus titrations were performed by plaque assay as described by Campbell and Colter (1965). L cells, in BME-5% FCS, were grown in 60x15mm plastic petri dishes (Lux Plasticware) at 37° in a humidified atmosphere of 5% CO_2 in air until confluent monolayers were formed. After removal of the medium by aspiration, plates were inoculated with 0.2ml volumes of appropriate dilutions of virus in virus diluent. After incubation in air for 1 hour at 37° to allow virus to attach, plates were overlaid with 4.5ml aliquots of agar overlay, and incubated at 37° for an additional 48-72 hr in an atmosphere of 5% CO_2 in air. Plates were stained by the addition to each of 3 ml aliquots of agar overlay containing 0.01% neutral red (Fisher Scientific). Plaques so visualized

were counted 2-3 hr later.

Production and Isolation Of Hybridomas

Preparation of Spleen Cells

Spleens from immunized female BALB/c mice, which have histocompatibility antigens identical to those on Sp2/0-Ag14 myeloma cells, were removed 3 days after a final intraperitoneal booster injection of immunogen. Each spleen was placed in a 60mm plastic petri dish in warm (37°) DME-10% FCS and gently teased apart with two 22-gauge needles. The cells were washed into a 15ml Corning conical centrifuge tube and pelleted by centrifugation at 250xg for 7 min. Contaminating erythrocytes were lysed by resuspending the pellet in 5ml cold 0.17M NH_4Cl and placing the tube on ice for 10 min. The cells were then washed twice with 10ml of serum-free DME, counted and checked for viability by trypan blue (Fisher Scientific - 0.4% final concentration in PBS) exclusion. The yield of cells was approximately 10^8/spleen, and viability was routinely > 95%.

Fusion Method

Sp2/0-Ag14 myeloma cells were grown for several days prior to fusion in DME-10% FCS without 8-azaguanine. The fusion protocol employed was adapted from the method described by Gefter *et al.* (1977). Spleen lymphocytes were mixed with Sp2/0-Ag14 myeloma cells at the optimal ratio of 2 splenocytes:1 myeloma cell (Gerhard *et al.*, 1980), and

pelleted by centrifugation at 600xg for 8 min. The supernatant was aspirated and the pellet gently resuspended in 1ml of warm (37)° 30% polyethylene glycol (PEG 1000-Sigma) in DME, pH 7.6. Cells were immediately pelleted from the suspension by centrifugation at 600xg for 4 min. After an additional 4 min at room temperature the supernatant was removed and the cells were resuspended in 10ml of serum-free DME. After low speed centrifugation the pelleted cells were resuspended in 30ml of HAT selective medium (DME-10% FCS supplemented with 0.1mM hypoxanthine, 0.01mM aminopterin and 0.03mM thymidine; all obtained from Sigma) in which only hybridoma cells survive. The cells were dispensed in 50µl aliquots (containing approximately $3x10^5$ cells) into 6 Costar 96-well tissue culture dishes (Bellco Biological Glassware) and incubated at 37°. After 24 hours an additional 50µl of HAT medium was added to each well. On day 5 each well received another 100µl of HAT medium, and at 5-day intervals thereafter the HAT medium was charged in all wells.

Enzyme-linked Immunosorbant Assay (ELISA)

Hybridomas were screened for specific antibody production by ELISA 2 to 3 weeks after fusion, the procedure used being that described by Voller *et al.* (1976). Polystyrene 96-well ELISA plates (Dynatech Laboratories, Fisher Scientific Co.) were sensitized by adding to each well 100µl of the appropriate antigen in coating buffer (13.5mM Na_2CO_3, 34.9mM $NaHCO_3$, 0.02% sodium azide, pH 9.6)

and incubating for 24 to 48 hours at 4°. The wells were then washed three times with 200µl volumes of the phosphate-buffered saline containing Tween 20 (PBS-Tween) described by Voller *et al.* (1976). Hybridoma cell culture supernatants (100µl volumes) were then added to individual wells and the plates incubated in a humidifed chamber for 2 hours at room temperature. The wells were then emptied and washed as before, after which 100µl of alkaline phosphatase - conjugated rabbit anti-mouse immunoglobulin (1/300 dilution in PBS-Tween) obtained from Miles-Yeda Ltd., Rehovot, Israel, was added to each well. The plates were again placed in a humidified chamber and incubated for 2.5 hours at room temperature. Following a final series of washes with PBS-Tween, 100µl aliquots of p-nitrophenyl phosphate solution (Sigma-104 phosphatase substrate tablets, 1mg/ml in 10% diethanolamine buffer, pH 9.8) were added to the wells. The plates were incubated at room temperature, and at appropriate times were monitored for color (yellow) development using a Titertek multiscanner (Flow Laboratories) at 405 nm to identify hybridomas producing potentially useful monoclonal antibodies.

Cloning by Limiting Dilution

Hybridomas that were scored as positive by ELISA were transferred to new 96-well plates in HT medium (DME-10% FCS supplemented with 0.1mM hypoxanthine and 0.03mM thymidine) and re-tested after a period of 3 to 5 days. Hybridomas that remained ELISA-positive were then cloned at least twice by

limiting dilution. In this technique an appropriate dilution of cells from a positive well were dispersed in the first row of 8 wells in a 96-well cluster. In subsequent rows the cells were serially diluted in steps of 1/2 or 1/3 to an estimated end-point of 0.5 cell/well. Addition of mouse erythrocytes ($1x10^7$/ml) to each well prior to the addition of the hybridoma cells proved to be only marginally better than cloning without a feeder layer. After 2 to 3 weeks, wells that contained a single clone were tested by ELISA and positives were again cloned to ensure clonality. Hybridomas found to be producing specific monoclonal antibodies were transfered into 24-well plates (Linbro, Flow Laboratories) in DME-10% FCS and grown at 37° to obtain quantities of antibody sufficient for characterization.

Freezing Clones

Cells from each cloned hybridoma line were obtained from healthy, growing cultures and pelleted by centrifugation at 250xg. The pelleted cells were resuspended in ice-cold 5% dimethylsulfoxide (DMSO, Fisher Scientific) in FCS at a concentration of 1 to $2x10^6$ cells/ml. Several aliquots (1ml) of each suspension were frozen overnight by incubation at -70° and then transfered to liquid nitrogen.

Immunodiffusion

The immunoglobulin class and subclass of the monoclonal antibody produced by each cloned hybridoma line was determined by the Ouchterlony double diffusion technique

(Ouchterlony, 1948) using agar gel plates obtained from Hyland Diagnostics, Deerfield, IL. The central well was filled with hybridoma cell culture supernatant (7µl) and surrounding wells with rabbit antisera (7µl) to specific mouse immunoglobulins (Miles Laboratories Inc., Elkhart, IN.). The plates were incubated in a humidified chamber for 2 to 3 days at room temperature. Following incubation, the gel was separated from the plate and washed overnight in PBS. Immunoprecipitin bands were visualized by staining the gel with 0.2% Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Richmond, CA.) in 7% acetic acid -45% methanol and then destaining it in 7% acetic acid -40% methanol.

Growth of Hybridomas in Mice

Female BALB/c mice were primed by the intraperitoneal injection of 0.5ml pristane (2,6,10, 14-tetramethylpentadecane, Sigma) 1 to 7 weeks prior to the injection, by the same route, of hybridoma cells ($1x10^6$ cells in 0.5ml PBS). After 10 to 14 days ascites fluid was collected and allowed to clot at room temperature for 1 hour. Cells were removed by centrifugation at 3000xg for 10 min and the clarified ascites was stored at -20°.

Purification of Monoclonal Antibody from Ascites Fluid

Ascites fluid was filtered through a Millipore HA filter (pore size =0.45µm) before an aliquot (10 to 15 ml) was chromatographed at room temperature on a 2.6x90cm Sephacryl superfine S-200 (Pharmacia Canada Ltd., Dorval,

P.Q.) gel exclusion column that had been equilibrated with S-200 buffer (50mM Tris-HCl pH 8.0, 0.5M NaCl and 1mM EDTA). After sample application, the column was washed with the same buffer at a flow rate of 0.23ml/min and the wash monitored with a UV-1 Single Path Monitor (Pharmacia) at 280nm. Fractions (2.3ml) were collected, and those corresponding to the well-resolved major protein peak containing the immunoglobulin G (IgG) were pooled. To concentrate the IgG, pooled fractions were made 50% saturated with respect to ammonium sulfate (Ultra Pure, Canadian Scientific Products Ltd., London, Ont.), stirred overnight at 4° and then centrifuged for 30 min at 15000xg and 4°. The pelleted IgG was dissolved in a small volume of 0.3M NaCl-0.2M $NaHCO_3$ (pH 8.0), dialyzed against the same buffer overnight at room temperature, and stored at -70°. Protein concentrations of IgG preparations were estimated from A_{280} measurements, made with a Beckman Acta II spectrophotometer and assuming the extinction coefficient of IgG to be A_{280} (1mg/ml) = 1.5 (Mamet-Bratley, 1970). Isolated IgGs were analzyed by electrophoresis in 10% polyacrylamide slab gels containing 1% SDS, 2.5M urea and 0.1M sodium phosphate, pH 7.2, with a running buffer of 0.1M sodium phosphate, pH 7.2, containing 0.1% SDS and 0.1M 3-mercaptopropionic acid. Two bands, essentially free of contaminants, and with apparent molecular weights of 50,000 and 25,000, as expected for the heavy and light chains of IgG molecules (Eisen, 1980), were visualized by staining with Coomassie Brilliant Blue R-250 as

described above.

Purification of Monoclonal Antibodies from Hybridoma Cell Culture Supernatants

Cell culture supernatants (250ml) containing monoclonal antibodies were clarified by centrifugation at 250xg for 15 min and filtration through a Millipore HA filter (pore size - 0.45μm). Purified IgGs were isolated from such clarified supernatants by affinity chromatography on a protein A-sepharose CL-4B (Pharmacia) column (volume=1.5ml) which had been equilibrated and washed with 100mM Tris-HCl, pH 8.0. The bound antibody (approximately 5mg from 250ml culture medium) was eluted with 1M acetic acid, the eluate being monitored at 280nm. Fractions containing the IgG were pooled, dialyzed extensively against dilute (1/10) PBS in distilled water at 4° and stored at -20°. Protein concentrations were determined from A_{280} measurements as described above.

Preparation of F(ab) Fragments

Purified IgG (2mg) from hybridoma cell culture supernatants were dialyzed against distilled water and lyophilized. The lyophilized powder was dissolved in 1ml 100mM sodium phosphate, pH 7.2, 10mM cysteine-HCl and 2mM EDTA, and digested with papain (200μg/ml; Sigma) for 18 hours at 37° according to the procedure described by Porter (1959). The F(ab) fragments were purified by the method described by Emini *et al*. (1982). The papain digest was

applied to a 0.8x30cm Sephadex G-75 (Pharmacia) gel filtration column at room temperature to remove papain. The column was equilbrated and washed with 150mM NaCl - 100mM Tris-HCl, pH 8.0, at a flow rate of 0.25ml/min. The flowthrough was monitored at 280nm and fractions corresponding to the initial protein peak were passed through a protein A-sepharose CL-4B affinity column (1.5ml) in order to remove undigested IgG and F_c fragments. The flowthrough, containing pure F(ab) fragments, was dialyzed extensively against distilled water at 4°. Protein concentrations were determined using a Bio-Rad protein assay kit with bovine plasma gamma globulin (Bio-Rad) as the standard. Analyses by electrophoresis on 10% polyacylamide slab gels as described above, revealed a single band (in some cases resolved into a doublet) with an apparent molecular weight between 20,000 and 25,000 as expected for F(ab) fragments (Eisen, 1980).

CHAPTER III

Antigenic Determinants on Mengo Virus: An Analysis Using Monoclonal Antibodies

Introduction

Studies of picornaviral antigens and of the mechanism(s) of virus neutralization have evolved slowly due to a number of misconceptions based on relatively limited data. The use of a variety of animal species in which to raise polyclonal sera and the use of different antigen preparations and different means of presenting the antigens to the animals have contributed to the generation of contradictory results. With the advent of monoclonal antibody technology (Köhler and Milstein, 1975) a more refined look at viral antigenic determinants and mechanisms of viral neutralization became possible. Animal sera have provided information on immunodominant viral antigens and little or no information on precise viral epitopes involved in neutralization.

Monoclonal antibodies have been used to identify neutralizing antigenic determinants on poliovirus VP1 (Emini *et al.*, 1982, 1983a; Minor *et al.* 1983; Wychowski *et al.*, 1983; Blondel *et al.*, 1983; Evans *et al.*, 1983;), FMDV VP1 (Baxt *et al.*, 1984; Robertson *et al.*, 1984; Duchesne *et al.*, 1984); human rhinovirus VP1 and VP3 (Sherry and Rueckert, 1985); and Theiler's murine encephalomyelitis virus VP1 (Nitayaphan *et al.*, 1985). These results coupled with

reports of the ability to elicit neutralizing antisera with isolated poliovirus VP2 and VP3 (Dernick *et al.*, 1983; Emini *et al.*, 1984; coxsackie virus VP2 (Beatrice *et al.*, 1980); and Mengo virus polypeptide α (Lund *et al.*, 1977) suggest very strongly that virus neutralization may be achieved by several distinct mechanisms.

Synthetic peptides have also been used to elicit neutralizing antisera against epitopes on FMDV VP1 (Bittle *et al.*, 1982; Pfaff *et al.*, 1982; Geysen *et al.*, 1984, 1985) and poliovirus VP1 (Emini *et al.*, 1983c). The primary objective of these investigations has been the development of potent, more stable and perhaps safer vaccines for FMDV and poliovirus. Mengo virus is not an agent of either economic or public health importance, but it does provide a model system for studies of picornaviral neutralization.

Only a handful of investigators have begun to come to grips with the actual mechanism(s) of viral neutralization, and of these only a few have carried out such investigations in a strictly quantitative manner (with known antibody molecule:virion ratios for example). The results detailed in this chapter show that subviral (13.4S) particles derived from pH-inactivated Mengo virus possess neutralization epitopes. Two monoclonal antibodies specific for polypeptide β have been shown to be capable of neutralizing Mengo virus by what appear to be two different mechanisms. One (MCP-6) at very low antibody:virion ratios is able to neutralize by

simple aggregation of intact virions. A second monoclonal antibody (MCP-5) appears to neutralize the virus by blocking its attachment to the cellular receptor without inducing significant aggregation of virions. This appears to be the first report of an anti-polypeptide β antibody that acts in this way.

Materials and Methods

Establishment of Hybridoma Lines Producing Monoclonal Antibodies

Cells obtained from the spleens of 6 immunized BALB/c mice were used in two separate fusions during the course of this study. The mice were immunized by the injection of highly purified Mengo virus which had been dissociated by dialysis against PBS-HCl, pH 6.2, for 24 hr. at 37°, the principal protein product of the dissociation being a 13.4S particle having the composition $(\alpha, \beta, \gamma)_s$ (Mak *et al.*, 1971, 1974). Electron microscopic examination of negatively stained (1% sodium phosphotungstate) samples of the dialysate revealed the presence of 13.4S particles (and essentially total absence of intact virions), and infectivity (plaque) assays of the preparations confirmed that inactivation of the virus was >99%.

Each mouse was injected by the intraperitoneal (i.p.) route with 10μg of viral protein (as estimated by the Bio-Rad Bradford assay) suspended in 200μl Freund's complete adjuvant (Difco), followed, at 2 week intervals, by booster

injections (i.p.) of the same quantity of viral protein suspended in Freund's incomplete adjuvant (Difco). Fusions were performed 3 days following the second or third booster injection as described in Chapter II. One day prior to the fusion event, sera obtained from the immunized mice by tail bleeding were tested for the presence of specific antibody by the ELISA, using Dynatech substrate plates sensitized with pH-inactivated Mengo virus (approx. 1µg/well). Spleens from responding mice were used. Hybridoma cell culture supernatants were screened for the presence of antibody by the same technique, using the same antigen preparation. ELISA-positive hybridomas were cloned at least twice by the limiting dilution technique.

Infection of Monolayers and Labeling of Viral Polypeptides

To prepare cell lysates that were used in immunoprecipitation studies of the monoclonal antibodies, L cell monolayers grown in petri dishes (100mm diameter, Lux Plasticware) were infected with Mengo virus suspended in virus diluent at an estimated multiplicity of 100 PFU/cell. After incubation for 1 hr at 37°, the monolayers were washed with warm (37°) BME-1% FCS and then incubated at 37° in the same medium. At 5 hr post-infection this medium was replaced by methionine- and glutamine-free MEM (Flow Laboratories), and incubated for an additional 1 hr before the cells were pulse-labeled by incubation for 30 min in methionine- and glutamine-free MEM containing [^{35}S] methionine (>800 Ci/mmole, New England Nuclear) at a concentration of

100μCi/ml. Following the labeling period, the radioactive medium was removed and the monolayers were washed twice with ice-cold PBS, before being lysed in immunoprecipitation buffer (IB) composed of 150mM NaCl - 40mM sodium phosphate, pH 8.0, containing 1% NP-40 (Shell Oil of Canada Ltd.), 0.5% deoxycholate (BDH Chemicals Canada Inc.), 1mM phenylmethylsulfonyl fluoride (PMSF, Sigma) and aprotinin (Sigma) at a concentration of 500KIU/ml. Nuclei were pelleted by centrifugation at 10,000xg for 10 min at 4°, and the supernatant was removed carefully and used as the source of antigen(s) in the immunoprecipitation studies outlined below.

The limited cleavage studies described in this chapter, though peripheral to the main line of investigation, were suggested by certain results obtained from analyses of viral polypeptides immunoprecipitated from extracts of infected cells by the monoclonal antibodies described herein. In these studies, cells grown in petri dishes (60x15mm) were infected at 33° with a suspension of Mengo virus in virus diluent at a multiplicity of 20 PFU/cell. After incubation for 1 hr, the monolayers were washed with warm (37°) amino acid-deficient Eagle's medium containing actinomycin D (Merck, Sharp and Dohme Int., Rahway, NJ.; 5μg/ml) and incubated at 33° in the same medium. Viral polypeptides were labeled at 8.5 hr post-infection by incubating the infected-cell monolayers in amino acid-deficient medium containing actinomycin D and [4,5^3-H] leucine (61Ci/mmol;

Amersham) at concentrations of 5μg/ml and 100μCi/ml respectively. Labeling was carried out for 15 min at either 33° or at 39°. In the latter case, the temperature was increased from 33° to 39° for 30 min prior to the labeling period. When labeling was followed by a chase period, the radioactive medium was removed, the monolayers washed with warm PBS and incubated for 90 min in BME-5% horse serum (Flow laboratories) containing 5μg actinomycin D/ml.

Lysates were prepared by removing the medium after either the pulse or the chase period, washing the monolayers twice with ice-cold PBS, and then adding 0.4ml of warm (37°) lysis mixture (10mM sodium phosphate, pH 7.2, containing 2% SDS, 5% β-mercaptoethanol and 1mM PMSF). Aliquots of the cell lysates (containing approximately $5x10^5$ acid-insoluble cpm/aliquot) were supplemented with glycerol and bromophenol blue (final concentrations = 20 and 0.002% respectively) before being heated at 100° for 5 min and analyzed by SDS-10% polyacrylamide slab gel electrophoresis as described in Chapter II.

Immunoprecipitation of Virus-Specific Structures

A 2ml aliquot of the [^{35}S]-labeled infected cell lysate was incubated (with mixing) for 18 hr at 4° with 1 ml of Sp2/0-Ag14 myeloma cell culture supernatant, after which 1.5ml of a 10% suspension of formalin-fixed *S. aureus* (IgGSorb, The Enzyme Center, Boston, MA.) in IB containing 5mg ovalbumin/ml was added, and incubation was continued for

an additional 1 hr at 4°. After removal of the protein A-containing *S. aureus* by centrifugation, the supernatant, designated 'preadsorbed antigen' was made 0.1% with respect to SDS.

Aliquots (200µl) of preadsorbed antigen were incubated (with mixing and at 4°) with 100µl volumes of hybridoma cell culture supernatants. After 18 hr, a 100µl aliquot of a 10% suspension of IgGSorb in IB containing 0.1% SDS and 5mg ovalbumin/ml was added to each mixture and incubation was continued for 1 hr at 4°. The IgGSorb with attached antigen-antibody complexes was pelleted by centrifugation and washed twice with IB containing 0.1% SDS and made 0.5M with respect to NaCl, and once with IB containing 0.1% SDS. Washed pellets were resuspended in 60µl volumes of 10mM sodium phosphate, pH 7.2, containing 2% SDS, 5% β-mercaptoethanol and 0.002% bromophenol blue, and heated at 100° for 5 min. Free IgGSorb was pelleted by centrifugation and the supernatants were analyzed by electrophoresis in 10% polyacrylamide slab gels as described in Chapter II. Protein bands were visualized by fluorography (En³Hance; New England Nuclear) of dried gels using pre-flashed (Laskey and Mills, 1975) Kodak X-Omat AR film.

Determination of Monoclonal Antibody Specificity by Protein Blotting

Renaturing Western blots were prepared by a modification of the technique described by Bowen *et al.* (1980). Purified Mengo virus was solubilized by heating at 100° for 5 min in the presence of 1% SDS, 2.5% β-mercaptoethanol, and 0.002% bromophenol blue, after which the individual viral proteins were resolved by electrophoresis in 10% polyacrylamide slab gels using an estimated 150μg of viral protein per sample well. In some cases, lanes containing prestained high molecular weight protein standards (Bethesda Research Laboratories, Inc., Gaithersberg, MA.) were included. Following electrophoresis the slab gel (15x20x0.15cm) was immersed in 500ml of renaturation buffer (50mM NaCl-10mM Tris-HCl, pH 7.5, containing 2mM EDTA, 0.1mM DTT and 4M urea) for 1 hr. This and all subsequent manipulations were performed at room temperature. The gel was then soaked in fresh renaturation buffer (500 ml) for an additional 3 hr after which the partially renatured polypeptides were transfered onto nitrocellulose sheets (BA 85; Schleicher and Schuell) by contact diffusion, the transfer taking place over a period of 72 hr and in the presence of transfer buffer (50mM NaCl-10mM Tris-HCl, pH 7.5, containing 2mM EDTA and 0.1mM DTT) as described by Bowen *et al.* (1980). The transfer buffer was replaced with fresh buffer every 18 hr.

The radioimmunoassay was performed by a modification of the technique described by Smith and Summers (1981). Visual inspection of the transfered prestained molecular weight markers allowed alignment of the original gel lanes on the nitrocellulose sheet. One lane containing viral polypeptides was excised and transfer efficiency checked by staining the protein bands with 0.1% amido black 10B (E. Merck AG. Darmstadt, FGR.) in 45% methanol and 10% acetic acid for 5 min and destaining with deionized water. After marking the remaining gel lanes on the nitrocellulose sheet, the sheet was incubated in 500ml of blocking buffer (150mM NaCl-50mM Tris-HCl, pH 7.4, containing 5mM EDTA, 0.5% ovalbumin and 0.05% NP-40) for 20 hr. The nitrocellulose sheet was then cut into strips corresponding to the gel lanes. Individual strips were placed in 50ml Corning polycarbonate centrifuge tubes attached to a rotator and were incubated for 18 hr in 25ml of blocking buffer containing 0.2 to 1mg of purified monoclonal IgG. The nitrocellulose strips were then rinsed briefly with deionized water and washed with blocking buffer (200ml) for 5 hr in order to remove excess antibody, after which they were incubated with a solution of ^{125}I-labeled $F(ab')_2$ fragment of sheep anti-mouse Ig (Amersham; 7.6×10^6 cpm/25ml blocking buffer) for 2 hr. Finally, the nitrocellulose strips were washed for 72 hr with 8 changes (200ml each) of 150mM NaCl-50mM Tris-HCl, pH 7.4, containing 5mM EDTA and 0.1% NP-40 to remove excess ^{125}I-$F(ab')_2$, before being air-dried and exposed to Kodak X-Omat AR film

with a Cronex Lightning Plus Intensifying Screen (DuPont de Nemours and Co. Inc., Wilmington, DE) for 24 to 48 hr at -70°.

Effect of Monoclonal Antibodies on Viral Attachment

Aliquots of a suspension of highly purified, [^{3}H] uridine-labeled virus in virus diluent were mixed with monoclonal IgGs, purified from hybridoma cell culture supernatants and dissolved in virus diluent, at known antibody:virus ratios expressed as molecules of IgG/virus particle. Concentrations of stock IgG solutions were determined from A_{280} measurements as described in Chapter II, and virus concentrations were calculated from A_{260} measurements using the relationship, 1 A_{260} unit = 9.5×10^{12} virus particles/ml (Scraba, 1968). Incubations, in a total volume of 1ml, were for 2 hr at room temperature in Eppendorf tubes (1.5ml) which had been precoated with 1% FCS in PBS to minimize non-specific loss of virus by adsorption of either virions or virion-IgG complexes to the tubes.

Following the 2 hr incubation at room temperature, 200µl aliquots of each virus-antibody mixture were pipetted onto duplicate, confluent L cell monolayers (in 60mm petri dishes) which had been cooled to 4° and from which growth medium had been removed. After incubation for 1 hr at 4°, unattached virus was removed by washing each plate twice with 1ml volumes of PBS, which were pooled and added to scintillation vials. Monolayers with attached virus were

solubilized in 1ml of 2% SDS in 10mM sodium phosphate, pH 7.2, and transfered, together with a 1ml wash of solubilization buffer, into scintillation vials. Radioactivity in both the pooled PBS washes and the solubilized monolayers was measured in the presence of 15ml of ACS (Amersham) scintillation fluid containing 0.5% acetic acid (final concentration) using a Beckman liquid scintillation spectrometer (Model LS-330).

Sucrose Density Gradient Analyses

Aliquots (100µl) of the virus-antibody mixtures used in the attachment studies outlined above were layered on 4.8ml, 5-40% linear sucrose gradients (made in PBS containing 0.1% ovalbumin) and centrifuged at 35,000 rpm (SW 50.1 rotor; Beckman L8-80 ultracentrifuge) for 90 min at 5°. Gradient fractions (150µl) were collected directly into scintillation vials using a Beckman gradient fractionator linked to an LKB peristaltic pump and fraction collector. Radioactivity was measured as indicated above after the addition of 10ml of ACS scintillation fluid containing 0.5% acetic acid to each.

Neutralization of Virus Infectivity

An aliquot (10µl) of each of the virus-antibody incubation mixtures used in the attachment studies described above was assayed for infectious virus by the plaque assay described in Chapter II.

Results

Isolation of Hybridomas

Attempts to immunize BALB/c mice by the innoculation of 'sub-lethal' amounts of intact, purified virus were unsuccessful. Mice that were injected with 10^4 or more PFU of virus did not survive, while those that were challenged with less than 10^4 PFU did not respond (even after several booster injections) by producing detectable circulating antibodies against the structural polypeptides of Mengo virus.

However, a total of 6 hybridomas that produce monoclonal antibodies to viral structural polypeptides were isolated following the fusion of Sp2/0 cells with spleen cells from mice that had been immunized by the injection of pH (6.2)-inactivated virions. These cell lines, designated MCP-1 to 6 (for Mengo Capsid Polypeptide) are listed in Table 2, together with the class of immunoglobulin produced by each.

Specificity of Monoclonal Antibodies

Viral capsid polypeptides do not exist in monomeric form in extracts of Mengo-infected cells but are present exclusively in either progeny virions or sub-viral particles. That being the case, one would expect that a monoclonal antibody directed against a determinant in a single structural polypeptide would immunoprecipitate not

TABLE 2

Monoclonal Antibodies to Mengo

Structural Polypeptides

Hybridoma Line	Class of Immunoglobulin Produced
MCP-1	IgG_{2a}
-2	IgG_1
-3	IgG_1
-4	IgG_1
-5	IgG_1
-6	IgG_{2a}

one but several structural polypeptides from such an extract. That expectation was born out by an experiment the results of which are illustrated in Figure 2, a fluorogram of [^{35}S]-labeled polypeptides immunoprecipitated from an extract of infected cells by the monoclonal antibodies produced by hybridoma lines MCP-1 to 6, and resolved by SDS-PAGE. While all 6 monoclonal antibodies were found to immunoprecipitate several capsid polypeptides from the extract, the results also show clearly that there are differences among them with respect to specificity. Monoclonal antibodies MCP-2 and 3 immunoprecipitate 14S particles, $(\epsilon\alpha\gamma)_s$, but not intact virions from the extract, as shown by the presence of polypeptides ϵ, α and γ, and the virtual absence of polypeptides β and δ in the immunoprecipitates. Hybridoma MCP-2 may have a slight affinity for intact virions, since there appears to be a very faint band corresponding to polypeptide β. The other 4 monoclonal antibodies immunoprecipitate both 14S particles and intact virions from such extracts, as evidenced by the presence of polypeptides ϵ, α, β, γ and δ in the immunoprecipitates. Neither myeloma Sp2/0-Ag14 cell culture supernatant nor a monoclonal antibody against polyoma virus VP1 (PyCP-1) precipitated any radiolabeled polypeptides from these extracts (data not shown). Attempts to immunoprecipitate individual polypeptides from solutions of either SDS-solubilized (and denatured) virion polypeptides or formic acid solubilized virions (Dernick *et al.*, 1983) with monoclonal antibodies

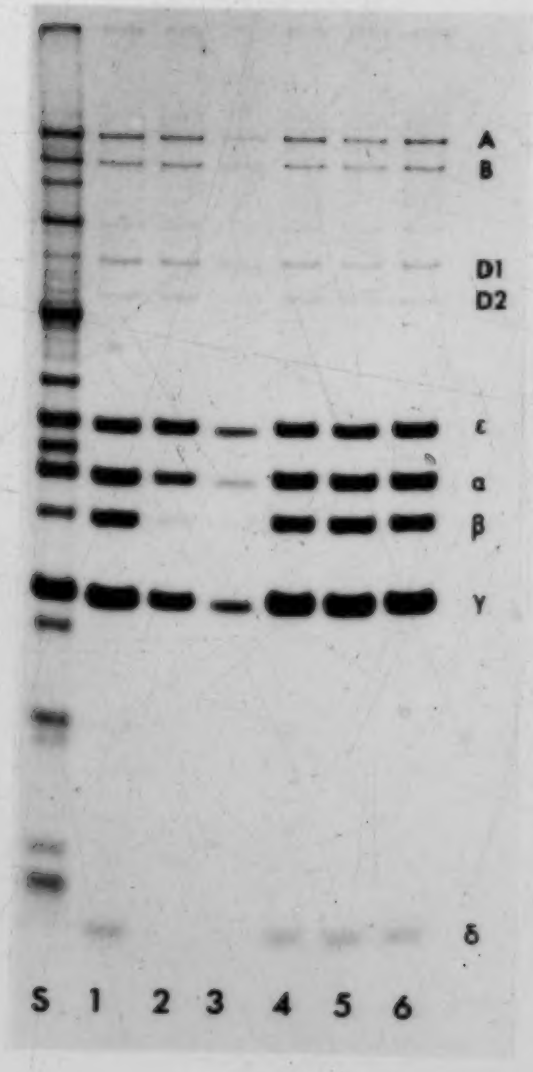

Figure 2. Fluorogram of [^{35}S]-labeled polypeptides immunoprecipitated from an extract of Mengo virus-infected L cells (lane S) by monoclonal antibodies produced by hybridomas MCP-1 to 6 (lanes 1 to 6), and resolved by electrophoresis in an SDS-10% polyacrylamide slab gel.

MCP-1 to 6 were unsuccessful, even after extensive dialysis of both preparations.

The fluorogram shown in Figure 2 reveals the presence in immunoprecipitates, not only of capsid polypeptides, but also of several precursor polypeptides previously described for Mengo virus (Paucha *et al.*, 1974). It is clear that immature, virus-specific structures containing precursor proteins A, B and D2, which on cleavage produce capsid polypeptides α, β, γ and δ (see Figure 1) are immunoprecipitated by all 6 anti-capsid monoclonal antibodies. In addition, the immunoprecipitates contain a polypeptide that has been designated D1 in Figure 2 by analogy with a protein of comparable molecular weight that has been identified in the EMC virus system (Butterworth *et al.*, 1971). The presence of this protein suggests the existence of a previously undetected cleavage pathway in the Mengo virus system, in which polypeptide D1 is the immediate precursor of polypeptides ϵ and γ. Perhaps a better demonstration of the presence of polypeptides D1 and D2 in Mengo infected cells is provided in the following chapter by Figure 7, which shows the polypeptide composition of high-affinity, virus-specific structures that are bound to and eluted from an immunoaffinity column bearing purified MCP-4 IgG.

Indirect confirmation of the existence of the alternate, D1 cleavage pathway has been obtained from classical pulse-chase experiments. Cells infected with Mengo virus at

33° were pulse-labeled for 5 min with ^{3}H-leucine at a time (8.5 hr post-infection) when host protein synthesis is almost completely shut off, thus permitting easy identification of virus-specific polypeptides. A typical fluorogram obtained from a pulse-chase experiment of this kind is shown in Figure 3. A comparison of the radiolabeled proteins present in infected cells immediately after a labeling period with those present after a subsequent 90 min chase period (compare lanes 1 and 2, or lanes 3 and 4) illustrates the flow of radiolabel from large precursor proteins to stable cleavage (viral gene) products. Polypeptides D1 and D2 are both present, most noticeably in lysates prepared immediately following the labeling period (lanes 1 and 3). It is of particular interest that in these lysates (i.e. those run in lanes 2 and 3) the amount of polypeptide α was found to exceed significantly the amount of polypeptide γ. Analysis of the fluorogram using a Joyce-Loebl Chromoscan 3 integrating densitometer (Vickers Instruments Inc., Malden, MA.) revealed that in the lysate made immediately after pulse-labeling for 15 min at 33°, the ratio of polypeptides α:γ was approximately 3.6:1, but that following a subsequent 90 min chase, the ratio was reduced to 1.3:1. The values arising from the densitometric analysis and presented in Table 3 were obtained by dividing the area (in arbitray integration units) of the individual peaks by the number of leucine residues present in each polypeptide chain (Ziola and Scraba, 1975).

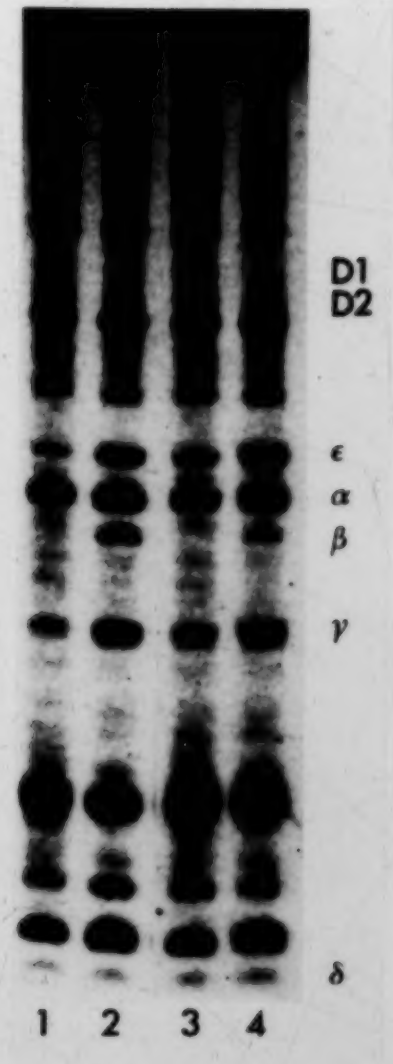

Figure 3. Electrophoresis in SDS-10% polyacrylamide slab gels of lysates of Mengo virus-infected cells, pulse-labeled for 15 min with [^{3}H]-leucine at 8.5 hr post-infection. Lane 1: cells were lysed immediately after the labeling period at 33°. Lane 2: cells were lysed after a subsequent chase period of 90 min at 33°. Lane 3: cells were shifted to 39° at 8 hr post-infection and lysed immediately after the labeling period at 39°. Lane 4: as in lane 3, except cells were lysed after a chase period of 90 min at 39°.

TABLE 3

Relative Quantities of Mengo Virus-Specific Polypeptides

Viral Polypeptide	Relative Quantities[a] 33° After pulse	33° After chase	39° After pulse	39° After chase
ϵ	32.7	168.9	92.4	238.1
α	294.6	484.9	333.9	438.8
β	28.4	132.9	20.2	61.6
γ	81.8	377.3	151.0	348.3

[a] Peak areas, determined by densitometric analysis of the fluorogram in Figure 3, were normalized to the number of leucines present in each polypeptide.

Results obtained from the pulse-chase experiment (illustrated in lanes 3 and 4 of Figure 3) in which cells infected at 33° were shifted (at 8 hr post-infection) to 39° for 30 min prior to pulse-labeling for 15 min (at 39°) were qualitatively (if not quantitatively) very similar to those obtained from the experiment carried out at 33°. The ratio of polypeptides α:γ in the lysate prepared immediately after pulse labeling for 15 min at 39° was only 2.2:1 whereas after the chase period at 39° the ratio was reduced to 1.3:1, similar to that seen at 33° (Table 3).

These results demonstrate clearly a significant departure from the expected 1:1 ratio of polypeptides α:γ if D2 $\rightarrow \alpha + \gamma$ were the exclusive cleavage pathway leading to the production of these molecules. The presence of the virus-specific polypeptide D1 with an apparent molecular weight of 65,000 suggests that an alternate cleavage pathway (D1 $\rightarrow \epsilon + \gamma$) similar to that described earlier for EMC virus (Butterworth *et al.*, 1971) may also exist in the Mengo virus system.

Additional efforts to identify the polypeptide carrying the antigenic determinant for each of these monoclonal antibodies were made using the technique described by Emini *et al.* (1982) and by which these investigators purported to identify neutralization epitopes on poliovirus. In brief, the method involved attempts to link isolated F(ab) fragments generated from the monoclonal antibodies to their

antigenic determinants on either intact or pH-inactivated radiolabeled virions using the heterobifunctional cross-linking reagent toluene-2,4-diisocyanate. The basis of the method is that when the mixture is solubilized following the reaction with F(ab) fragments to which the cross-linking reagent is attached, and analyzed by electrophoresis in SDS-12.5% polyacrylamide gels, those proteins which are cross-linked do not migrate into the gels. Theoretically then, the capsid polypeptide carrying the antigenic determinant for each monoclonal antibody may be identified by its exclusion from the gel. Repeated attempts failed to provide any meaningful data. With each monoclonal antibody examined, always two (α and γ) and sometimes three (α, β and γ) of the capsid polypeptides were partially or even totally excluded from the gel. Furthermore, essentially the same results were obtained when ovalbumin was substituted for the F(ab) fragments derived from the monoclonal antibodies. Exhaustive examination of the technique made it increasingly obvious that if any specific interactions did occur, they were completely obscured by extensive, non-specific cross-linking.

Some success at characterizing the anti-capsid monoclonal antibodies was achieved by the technique of renaturing Western blotting (Bowen *et al.*, 1980; Smith and Summers, 1981) as outlined in the Materials and Methods section of this chapter. Initial attempts to characterize the antibodies by conventional Western blotting using either diazophenylthioether (DPT) paper (Alwine *et al.*, 1977;

Bittner *et al.*, 1980; Honda, B., personal communication) or nitrocellulose sheets (Towbin *et al.*, 1979) were unsuccessful. The renaturation step (i.e. incubation in 4M urea) was found to be an absolute requirement for antibody binding. Using this method, the polypeptides containing the antigenic determinants for monoclonal antibodies MCP-2, MCP-5 and MCP-6 were shown clearly to be polypeptide α, polypeptide β and polypeptide β respectively (Figure 4). Despite repeated attempts, definitive results were not obtained with the remaining 3 anti-capsid polypeptide monoclonal antibodies, suggesting that they have a more strict conformational dependency for interaction with their determinants, possibly requiring that the polypeptides carrying those determinants remain associated with other capsid polypeptides in order to maintain the correct configuration. During the course of these studies it was determined that in terms of efficiency of blocking non-specific interactions the various agents in order of increasing efficiency were:gelatin (0.25% w/v) < BSA fraction V (0.5%) < ovalbumin (0.5%). FCS (10%) and sheep haemoglobin (1% in PBS) both appeared to inhibit specific as well as non-specific interactions.

The results indicate that monoclonal antibody MCP-2 recognizes an antigenic determinant on polypeptide α that is present and accessable in 13.4S particles (produced by dissociation of the virus particle at pH 6.2) and in 14S particles (found in infected cells), but which is either

Figure 4. Renaturing Western blot analysis of Mengo anti-capsid monoclonal antibodies using purified IgG from ascites: MCP-2 (250 µg), MCP-5 (200 µg) and MCP-6 (1 mg). ^{125}I-anti-mouse F(ab')$_2$ from sheep was used as the probe.

buried or has assumed a different conformation in the intact virion. Monoclonal antibodies MCP-5 and 6 each recognize an antigenic determinant on polypeptide β that is present and accessable in 13.4S and 14S particles as well as in intact virions.

Monoclonal Antibody-Virus Interactions

The attachment of [^{3}H] uridine-labeled Mengo virus to L cell monolayers in the presence of monoclonal antibodies was studied at antibody:virus ratios of 10, 30 and 60 IgG molecules/virus particle, and the results are summarized in Table 4. After 1 hr at 4°, at which temperature Mengo virus will attach to but not penetrate into L cells (Mak *et al.*, 1970), 61% of the control virus and 57% of virus incubated, at an antibody:virus particle ratio of 60, with a monoclonal antibody to VP1 of polyoma virus (PyCP-1) were found to be attached to the monolayers. Monoclonal antibodies MCP-1, 2 and 3 did not significantly affect viral attachment at any of the antibody:virus particle ratios employed. The results obtained with monoclonal antibodies MCP-2 and 3 were as expected, since neither is able to immunoprecipitate intact virions from suspension (see Figure 2).

In contrast, monoclonal antibodies MCP-4, 5 and 6 at an antibody:virus particle ratio of 60 (i.e. at antibody concentrations sufficient to saturate all 60 available epitopes on the virus particle) reduced viral attachment to approximately 40% of control values. However, inhibition of

TABLE 4

Interaction of Anti-Capsid Monoclonal Antibodies with Mengo Virus[a]

Monoclonal Antibody	Ab:particle ratio[b]	Attachment[c]	Sedimentation Behaviour[d]	Infectivity[e]
MCP-1	60	55	2.1	5.6
	30	61	19.4	23.6
	10	58	55.5	43.6
-2	60	62	100	100
	30	58	100	100
	10	58	94.2	100
-3	60	63	98.2	100
	30	57	100	100
	10	57	100	100
-4	60	23	0	0.6
	30	49	1.6	0.6
	10	85	0	1.4
-5	60	24	71.2	3.5
	30	31	83.6	21.8
	10	44	97.2	77.3
-6	60	24	0	1.7
	30	38	3.3	1.6
	10	78	0	2.6
PyCP-1[f]	60	57	98.4	100
None	-	61	100	100

[a] Virus-antibody mixtures and the control virus suspension (all containing the same quantity of ^{3}H-uridine virus) were incubated for 2 hr at room temperature before use in the attachment studies, for sedimentation analyses and for infectious plaque assays.

[b] Number of IgG molecules/virus particle.

[c] Per cent of input virus attached to L cell monolayers after 60 min at 4°.

[d] Per cent of virus that banded in the same position as control virions after centrifugation for 90 min at 35K rpm (SW50.1 rotor) in a 5-40% linear sucrose density gradient.

[e] Per cent of infectivity of control virus.

[f] Monoclonal antibody to capsid polypeptide VP1 of polyoma virus.

attachment was found to be very much dependent on antibody:virus particle ratios, since at lower ratios (10:1) the per cent of virus that became attached to the monolayers either approached (in the case of MCP-5) or exceeded (in the cases of MCP-4 and 6) the value obtained with the control (absence of antibody) virus.

Sucrose density gradient sedimentation analyses performed in conjunction with the viral attachment studies provided some explanation for the anomalous attachment results. Representative sedimentation profiles of the labeled virus-antibody mixtures are illustrated in Figure 5. The percentage of virus that banded in the position of marker monomeric virions is shown for all mixtures in Table 4. As expected, monoclonal antibodies MCP-2 and 3 had no effect on the sedimentation behavior of the virus. Monoclonal antibodies MCP-4 and 6 were the most active in cross-linking virus particles, even at antibody:virus particle ratios of 10, both produced large aggregates which were pelleted under the centrifugation conditions that were used. A possible explanation of the apparently enhanced attachment of virus to monolayers in the presence of low concentrations of these monoclonal antibodies may be that large aggregates of lightly cross-linked virus are able to attached with reasonably high efficiency to L cells. The effects of monoclonal antibodies MCP-1 and 5 the sedimentation behavior of the virus fall somewhere in between those of the other two pairs in that aggregation was found to be

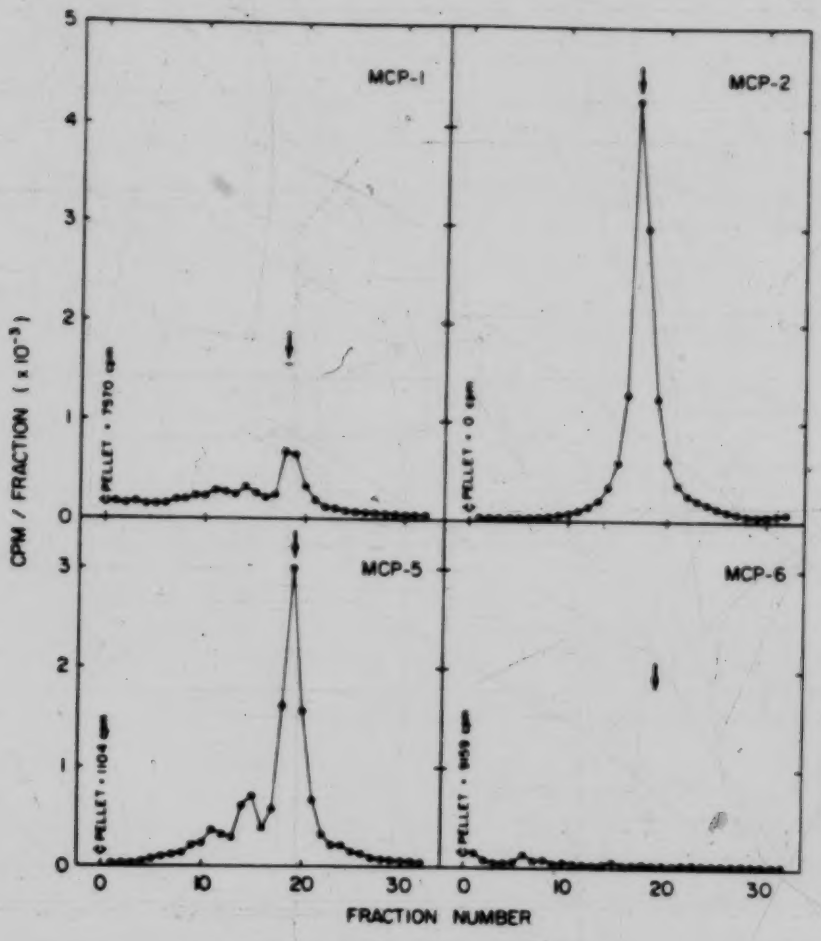

Figure 5. Sedimentation analysis of ^{3}H-uridine labeled Mengo virus after incubation at room temperature for 2 hr with monoclonal antibodies at antibody:particle ratios of 30(MCP-1), 60(MCP-2), 60(MCP-5) and 10(MCP-6). Centrifugation through a 5-40% sucrose linear gradient was for 90 min at 35,000 rpm in a SW50.1 rotor. Direction of sedimentation = right to left. Arrows indicate the position of marker Mengo virions.

very much dependent on the antibody:virus particle ratio over the range of 10 to 60.

Data regarding the neutralization of virus infectivity by the various monoclonal antibodies is also summarized in Table 4. The titer of the control virus was approximately 1.1×10^8 PFU/ml and the infectivity of each antibody-virus mixture is shown as a percentage of that value. Monoclonal antibodies MCP-2 and 3, as expected, had no affect on viral infectivity. The remaining four monoclonal antibodies MCP-1, 4, 5, and 6 neutralized infectivity efficiently at an antibody:virus particle ratio of 60. However, while monoclonal antibodies MCP-4 and 6 were strongly neutralizing at all antibody:virus particle ratios studied, indicating that large aggregates of virus are non-infectious, the neutralizing abilities of monoclonal antibodies MCP-1 and 5 were again very much dependent on antibody:virus particle ratios over the range of 10 to 60.

Discussion

During the course of these studies several reports in the literature (Icenogle *et al.*, 1981; Thorpe *et al.*, 1982; Brioen *et al.*, 1982; Meloen *et al.*, 1983) have described the difficulties encountered in characterizing the antigenic specificity of picornaviral neutralizing monoclonal antibodies by conventional techniques. The inability of neutralizing monoclonal antibodies to recognize denatured polypeptides has led to the development of alternate

methods, the most successful of which involves the isolation of non-neutralizable variants and comparing the sequence of their structural polypeptides to those of the wild-type proteins as deduced from sequence analysis of the viral cDNA (Minor *et al.*, 1983; Evans *et al.*, 1983). Unfortunately the Mengo virus genome has yet to be sequenced.

In summary, the data presented here show that monoclonal antibodies MCP-2 and 3 do not interact with intact virions and, as would be expected, have no effect on virus attachment, sedimentation behavior or infectivity. Binding of antibody to intact virions has been shown to be necessary but not necessarily sufficient for neutralization (Crainic *et al.*, 1983; Emini *et al.*, 1983a; Dimmock, 1984). The remaining four monoclonal antibodies neutralize viral infectivity and appear to fall into two groups; 1) those that neutralize primarily by causing aggregation of the virus (MCP-1, 4 and 6); and 2) those that neutralize primarily by blocking virus attachment (MCP-5).

The first group contains two monoclonal antibodies (MCP-4 and 6) which cause aggregation of the virus at very low antibody:particle ratios. It would seem likely therefore that the antigenic determinants recognized by both MCP-4 and 6 are located in such a position (greater than 150 Å apart; Werner *et al.*, 1972) that the antibody molecules tend to cross-link separate virions rather than enter into a bivalent attachment to a single virion. A similar mechanism

has been described for certain monoclonal antibodies to structural polypeptides of poliovirus (Brioen *et al.*, 1982; Thomas *et al.*, 1985) and FMDV (Baxt *et al.*, 1984). Even though the attachment data presented here indicate that large cross-linked aggregates are capable of enhanced, perhaps non-specific attachment to L cell monolayers, the large aggregates are clearly non-infectious. This suggests that they are either not internalized by the cells or are resistant to intracellular uncoating. Monoclonal antibody MCP-1 is less efficient than monoclonal antibodies MCP-4 and 6 in causing virus aggregation, and many favour bivalent attachment to the same virion. Thus virus neutralization by monoclonal antibody MCP-1 closely parallels the extent of aggregation.

The final neutralizing monoclonal antibody, MCP-5, appears to block viral attachment without causing extensive cross-linking (aggregation) of the virions. The data suggest that the position of the antigenic determinant on polypeptide β which is recognized by monoclonal antibody MCP-5 is such that monovalent attachment of the antibody to the virion is favored. Thus, even at high antibody:particle ratios there is not extensive aggregation (as shown in Figure 5, approximately 30% of the virions aggregate - as dimers or trimers - in the presence of 60 molecules of MCP-5 per virion), yet these high ratios are required in order to block attachment to any significant degree and thereby to neutralize virus infectivity. In light of those results

which show enhanced attachment of aggregates, one might speculate that the residual attachment that was observed with monoclonal antibody MCP-5 may have been due to attachment of non-infectious aggregates and/or non-specific adsorption.

The evidence presented here does not, however, make it possible to determine whether monoclonal antibody MCP-5 binds directly to the cell attachment site on the surface of the virion, or whether it binds to some other site in such a way as to sterically hinder binding to the receptor. Further meaningful studies on the neutralization of picornaviruses will require knowledge of the sequence and location on the surface of the virion of the antigenic epitopes involved in neutralization.

CHAPTER IV

Isolation of Pure Polypeptide E and Its Characterization as a Poly(U) Polymerase

Introduction

Following the development of an *in vitro* poly (A).oligo(U)-dependent polymerase assay for the poliovirus-specific polymerase by Flanegan and Baltimore (1977), attention has focussed on identification of the virus-specific and host components required for picornaviral RNA synthesis. The most extensively studied poliovirus-specific polymerase, designated either as P3-4b, p63 or NCVP4 (Flanegan and Baltimore 1977; Van Dyke and Flanegan, 1980; Baron and Baltimore, 1982c) and equivalent to Mengo virus polypeptide E (Lund and Scraba, 1979), acts strictly as an elongation polymerase requiring an RNA template and an oligo(U) primer for activity (Flanegan and Baltimore, 1977). A cellular protein, designated 'host factor' can substitute for an oligo(U) primer in the *in vitro* synthesis of minus strand viral RNA to produce a predominantly double-stranded product from the plus strand template (Dasgupta *et al.*, 1980, 1982; Baron and Baltimore, 1982a, 1982b). Unfortunately, polymerase preparations isolated by a series of chromatographic procedures contain contaminants which may constitute as much as 20% of their total protein content (Young *et al.*, 1985). These contaminants have been shown to include the polymerase precursor NCVP2, the 'host factor' for viral RNA synthesis (Andrews *et*

al., 1985), and Vpg-related polypeptides (Dasgupta *et al.*, 1983) which may possibly be involved in the initiation of viral RNA synthesis. Due to the presence of these impurities in polymerase preparations, the exact role(s) played by the 'host factor' and the Vpg-related polypeptides in the initiation of viral RNA synthesis remain somewhat obscure.

In this chapter, the purification of Mengo virus-specific polypeptide E to apparent homogeneity by immunoaffinity chromatography using an anti-polypeptide E monoclonal antibody is described. Results obtained using an *in vitro* poly(U) polymerase assay suggest that polypeptide E is the Mengo virus-specific elongation polymerase, and that the isolated polymerase is thermolabile. The observed heat-inactivation of polymerase activity can be correlated, by circular dichroism and fluorescence analysis with a conformational change in the enzyme.

Comparison of the wild-type poly(U) polymerase with those isolated from six RNA$^-$ ts Mengo mutants isolated in this laboratory (Downer *et al.*, 1976) suggests that the RNA$^-$ phenotype of 4 of the 6 ts mutants can be explained in terms of a temperature-sensitive lesion in their respective polymerases.

Materials and Methods

Virus

The wild-type virus used was the plaque variant of Mengo virus designated M-Mengo (Ellem and Colter, 1961). The isolation and partial characterization of mutants, ts 135, ts 506, ts 520, ts 620, ts, 625 and ts 677, from a mutagenized (nitrous acid) stock of M-Mengo have been described previously (Downer *et al.*, 1976).

Infection of Monolayers and Preparation of Cell Extracts

To prepare the cell lysates that were used to immunize BALB/c mice against Mengo-specific non-structural polypeptides, L cell monolayers grown in petri dishes (100mm diameter) were infected with Mengo virus suspended in virus diluent at an estimated multiplicity of 100 PFU/cell. After incubation for 1 hr at 37°, the monolayers were washed with warm (37°) BME-10% FCS and then incubated at 37° in the same medium. At 7.5 hr post-infection, the medium was removed and the monolayers were washed twice with ice-cold PBS before being scraped into reticulocyte standard buffer (RSB) composed of 10mM Tris-HCl, pH 7.5 containing 10mM KCl and 1.5mM $MgCl_2$. The cell suspension was made 1%, 0.5% and 1mM in NP-40, sodium deoxycholate and PMSF respectively and kept on ice with periodic mixing (vortex mixer) for 10 min. Nuclei were pelleted from the cell lysate by centrifugation at 10,000 xg for 10 min at 4° after which the supernatant was removed. Mengo structural polypeptides were removed by

immunoprecipitation with *S aureus* protein A (IgGSorb) complexed with MCP-5 monoclonal IgG. The protein A-IgG complex was prepared by incubating (with mixing at room temperature) a 20% suspension of IgGSorb in NET buffer (150mM NaCl-50mM Tris-HCl, pH 7.5, containing 5mM EDTA and 0.02% sodium azide) (Kessler, 1975) with an excess of anti-capsid (MCP-5) monoclonal antibody in the form of hybridoma cell culture supernatant. After 2 hr, the IgGSorb with attached antibody was pelleted by centrifugation and the pellet washed three times with NET buffer containing 0.1% BSA and 0.5% NP-40. The washed pellet was resuspended in the 10,000 xg supernatant from the lysate of Mengo-infected cells at a final concentration of 10% (v/v), and the mixture was incubated with mixing for 24 hr at 4°. The IgGSorb with attached antigen-antibody complexes was pelleted by centrifugation at 10,000xg for 10 min at 4°, and the supernatant was concentrated (approximately 5-fold) by vacuum dialysis (10,000 MW cutoff Collodion bags, Schleicher and Schuell) against PBS on ice.

Cell lysates used for the isolation of Mengo-specific poly(A).oligo(U)-dependent poly(U) polymerase (polypeptide E) were prepared from L cell monolayers grown either in 150cm² tissue culture flasks (Falcon Plastics) or in roller bottles and infected at an estimated multiplicity of 5-20 PFU/cell with either *wt* (at 37°) or ts Mengo virus (at 33°) as described above. When cytopathology was plainly visible (at 7.5-9 hr post-infection with *wt* virus at 37°, and 10-12

hr post-infection with ts RNA⁻ mutants at 33°) the medium was removed and the monolayers were washed twice with ice-cold PBS before being lysed with IB. Nuclei were pelleted as described above and virus particles were pelleted from the supernatants (S_{10}) by centrifugation at 43,000 rpm (Beckman 70 Ti rotor) for 2 hr at 4°. The supernatants (S_{43}) were removed carefully and stored at -70°.

Establishment of Hybridoma Lines Producing Monoclonal Antibodies

Cells obtained from the spleens of 5 immunized BALB/c mice were used in two separate fusions. The mice were immunized by the injection (i.p.) of concentrated Mengo-infected cell lysate (equivalent of 5-10x10⁶ cells) from which the capsid polypeptides had been removed by immunoprecipitation, suspended in 300µl Freund's complete adjuvant, followed, at two week intervals, by booster injections (i.p.) of the same quantity of lysate suspended in Freund's incomplete adjuvant. Fusions were performed 3 days following the fourth booster injection as described in Chapter II. Mouse sera and hybridoma cell culture supernatants were screened for the presence of antibody by the ELISA technique, using Dynatech Immulon 2 plates sensitized with S_{10} infected cell lysate (lysates processed only as far as the removal of cell nuclei) dialyzed into coating buffer.

Determination of Monoclonal Antibody Specificity by Immunoprecipitation

Immunoprecipitations of ^{35}S-labeled Mengo virus-specific polypeptides by hybridoma cell culture supernatants were performed as described in Chapter III except that antigen preparations (S_{10}) were preadsorbed with ascitic plasma (1/10 volumes) obtained by growing the MCP-4 (anti-capsid) hybridoma cell line in ascites form in BALB/c mice.

Immunoaffinity Purification of Mengo Virus Poly(U) Polymerase (Polypeptide E)

Anti-capsid polypeptide (MCP-4) and anti-polypeptide E (MNCP-20) immunoaffinity columns were prepared by mixing 20mg of IgG purified from ascites fluid with 1 ml of Affi-Gel 10 (Bio-Rad Laboratories), an N-hydroxysuccinimide ester of agarose to which the IgG couples via its free amino groups (Cuatrecasas and Parikh, 1972), and incubating for 4 hr at 4°. Unreacted active esters were blocked by adding 1 M ethanolamine-HCl, pH 8.0 (0.1ml/ml of gel) to the mixture and incubating at room temperature for 1 hr. The gel-immobilized antibody was washed with 150mM NaCl-40mM sodium phosphate, pH 8.0, and transferred into pasteur pipette columns. The columns were washed with elution buffer (150mM NaCl-0.2N acetic acid, pH 2.5, containing 0.5% NP-40) and then equilibrated with 150mM NaCl-40mM sodium phosphate, pH 8.0.

S_{20} supernatants (100-125ml) prepared from lysates of infected cells were passed, at a flow rate of 0.1-0.2ml/min at 4°, through an anti-capsid polypeptide 'pre-column' and then through a directly-attached anti-polypeptide E column. The two immunoaffinity columns were then uncoupled, and the anti-polypeptide E column was washed, at a flow rate of 0.5ml/min at 4°, with 40 volumes of 150mM NaCl-40mM sodium phosphate, pH 8.0, containing 1% NP-40 followed by 40 volumes of 150mM NaCl-sodium phosphate, pH 8.0 containing 2M KCl, before the bound protein was eluted with elution buffer. Fractions (1ml) of the eluate were neutralized with 175µl volumes of 3M Tris-HCl, pH 8, before being dialyzed overnight at 4° into replicase buffer (Baron and Baltimore, 1982a) composed of 50mM Tris-HCl, pH 8.0, containing 5mM β-mercaptoethanol, 0.5% NP-40 and 20% glycerol (v/v).

Aliquots (25µl) of the dialyzed fractions were made 2% with respect to SDS and 5% with respect to β-mercaptoethanol, heated at 100° for 5 min and analyzed by electrophoresis in SDS-10% polyacrylamide slab gels as described in Chapter II. Known quantities (1, 3, 5 and 10µg) of highly purified bovine serum albumin and ovalbumin (both obtained from Sigma) were electrophoresed simultaneously. Protein bands were stained with Coomassie Brilliant Blue R-250 as described in Chapter II and analyzed using a Joyce-Loebl Chromoscan 3 integrating densitometer. Protein concentrations in the dialyzed aliquots were estimated from a standard curve generated from the average of the densities

obtained with the two standard proteins.

Polymerase preparations used for circular dichroism and fluorescence measurements, and for amino acid and N-terminal sequence analyses were prepared in the same manner with the following modifications. The polymerase was eluted from the immunoaffinity column with 150mM NaCl-0.2N acetic acid, pH 2.5, containing 0.02% (v/v) octaethylene glycol ($C_{12}E_8$, NiKKol BL-8SY, NiKKō Chemicals Co., Ltd., Tokyo, Japan) a nonionic, ultra-violet transparent detergent (Helenius *et al.*, 1979). Fractions were dialyzed overnight at 4° into 50mM sodium phosphate, pH 7.0, before being analyzed as described above. Under these conditions, some of the protein precipitated from the peak fractions and was collected by centrifugation, pooled and washed with deionized water before being used in the amino acid and N-terminal sequence analyses.

Poly(U)polymerase Assay

The poly(A).oligo(U)-dependent poly(U) polymerase activity was assayed by a modification of the method described by Flanegan and Baltimore (1977). A 250µl aliquot of polymerase solution containing 4.0µg of enzyme was mixed with 250µl of standard assay mixture at 4° and incubated for 5 min at the assay temperature (33° or 39°) before the addition of 100µl of [5,6-^{3}H]UTP (New England Nuclear; 500-700 cpm/pmole). The final concentrations of reagents in the assay mixture were: 50mM Hepes-KOH, pH 8.0, 2mM magnesium

acetate, 10mM dithiothreitol (DTT), 60μM [^{3}H]UTP, poly(A) (Sigma; 20μg/ml) and oligo(U)$_{7-25}$ (10μg/ml). At appropriate time intervals, duplicate 60μl aliquots (containing 0.4μg of polymerase) were precipitated in 7% trichloroacetic acid (TCA) - sodium pyrophosphate (1/3 saturated) with 100μg of added carrier tRNA (Boehringer-Mannheim Canada, Dorval, P.Q.). The TCA-insoluble material was collected on HA membrane filters (Millipore, pore size=0.45μm), which were then washed extensively with 7% TCA and dried under a heat lamp for 30-60 min. Radioactivity collected on the filters was measured in Bray's scintillation fluid (New England Nuclear) using a Beckman Model LS-330 scintillation spectrometer.

Oligo(U)$_{7-25}$ was prepared by incubation of poly(U) (Sigma; 10μg/ml) for 3.5 hr at room temperature in 0.1 N NaOH. The digestion mixture was neutralized with 1N HCl before being applied to a Sephadex G-100 column that had been equilibrated with 10mM Tris-HCl, pH 7.5, containing 1mM EDTA. Peak fractions (monitored at 260nm) eluted in the same buffer were analyzed by electrophoresis on 20% polyacrylamide slab gels (Maniatis *et al.*, 1982), stained with 0.2% toluidine blue O (Fisher Scientific) in 0.2M sodium acetate, pH 4.7 (Grierson, 1982), and destained in deionized water to visualize the oligonucleotide bands. Fractions containing 7 to 25-mer oligo(U), as determined from the position of marker oligoribouridylic acid hexamer ((Up)$_5$U; Pharmacia P-L Biochemicals, Dorval, P.Q.), were pooled and the

oligonucleotides precipitated therefrom by the addition of 0.1 volume of 3M sodium acetate, pH 5.2, and 2 volumes of 95% ethanol. The 3' phosphate residues were removed by treating the oligo$(U)_{7-28}$ (1mg) with 100 units of calf-intestinal phosphatase (Boehringer-Mannheim) in 0.1M glycine, pH 10.2, containing 1mM $ZnCl_2$ and 1mM $MgCl_2$ for 45 min at 37°. The solution was extracted with phenol:$CHCl_3$:isoamylalchol:p-hydroxyquinoline (50:50:1:0.8%), the Oligo$(U)_{7-28}$ ethanol precipitated as before and resuspended in 10mM Tris-HCl, pH 7.5 containing 1mM EDTA at a concentration of 1mg/ml (1 A_{282}=50μg).

Amino Acid and N-terminal Sequence Analyses

Amino acid analyses of the immunoaffinity-purified polypeptides E were performed by Mike Natriss in the laboratory of Dr. L.B. Smillie. Aliquots containing 1 nmole of polypeptide E suspended in distilled water were lyophilized in 10x75mm pyrex tubes. The samples were acid hydrolyzed in 6N HCl at 110° in evacuated, sealed tubes for periods of 24, 48, 72 and 96 hr (Moore and Stein, 1954). The hydrolysates were lyophilized and the amino acids dissolved in 0.1ml of 0.2M sodium citrate, pH 2.2 before being resolved using a Durrum Model D-500 amino acid analyzer. The values of serine, threonine, valine and isoleucine were determined by extrapolation of the amino acid/alanine or amino acid/leucine ratios to zero time (Hirs *et al.*, 1954).

N-terminal sequence analyses of the *wt* and ts 520 polypeptides E were performed by automated Edman degradation using an Applied Biosystems gas-phase sequencer (model 470A). The analyses were done by Mike Carpenter in the laboratory of Dr. L.B. Smillie.

Circular Dichroism

Analyses of the immunoaffinity-purified polypeptides E by ultraviolet circular dichroism (CD) were carried out by K. Oikawa in the laboratory of Dr. C.M. Kay. CD measurements were made on a Jasco-500C spectropolarimeter equipped with a DPN-500N data processor and a thermostated cell holder. Baselines for the spectra were established at the initial temperature (20°) with 50mM sodium phosphate, pH 7.0 containing 0.02% octaethylene glycol ($C_{12}E_8$). For each sample, spectra were measured at increasing temperatures from 20° to 40° (using approximately 5° increments) following equilibration of the sample cell at each temperature of measurement. Relative ellipticities were calculated from the following equation:

$$\theta_{rel} = \frac{\theta_t}{\theta_{t\ initial}}$$

Where θ = observed ellipticity at a given temperature and $\theta_{t\ initial}$ =observed ellipticity at the initial (lowest) temperature. In this manner the concentration term in the calculation of molar ellipticities (Oikawa *et al.*, 1968) was effectively eliminated. The α-helix, β-sheet and random coil components were determined as described by Chen *et al.*

(1974) (See Appendix I).

Fluorescence

Tryptophan fluorescence measurements of the immunoaffinity-purified polypeptides E were performed by K. Oikawa in the laboratory of Dr. C.M. Kay. Excitation was at 280nm and the fluorescence at 333nm was measured with a Perkin-Elmer MPF-44B fluorescence spectrophotometer equipped with a thermostated cell holder. Baseline measurements were obtained using 50mM sodium phosphate, pH 7.0 containing 0.02% octaethylene glycol. Fluorescence measurements were obtained at increasing temperatures from 20° to 40° (using approximately 5° increments) following equilibration of the sample cell. To study the thermal behavior of the fluorescence, it is convenient to treat the data generated for each of the polypeptides as a function of temperature by means of Arrhenius plots (Brand and Witholt, 1967) employing the equation:

$$\frac{F_o}{F_t} - 1 = Ae^{\frac{-E}{RT}}$$

where F_o = initial fluorescence, F_t = fluorescence at T, A = pre-exponential factor, E = Arrhenius (empirical) energy of Activation (cal/mole) calculated from a plot of ln $(F_o/F_t - 1)$ versus 1/T, R = gas constant (1.987 cal/deg.mole), and T = temperature in degrees Kelvin. If there are differences in fluorescence properties of the system at high versus low temperatures, a plot of ln $(F_o/F_t - 1)$ versus 1/T will be characterized by two linear regions of different slope

(related to the activation energy of each form), and the point of intersection (break point) will be the transition temperature between the two forms. On the other hand, a simple monotonic loss of fluorescence with temperature is indicative of the absence of discrete temperature-dependent conformational forms.

Results

Isolation and Characterization of Antibody-Producing Hybridomas

Two hybridomas that produce monoclonal antibodies to viral non-structural polypeptides were isolated following the fusion of Sp2/0 cells with spleen cells from mice that had been immunized by the injection of Mengo-infected cell lysates from which the capsid polypeptide had been removed by immunoprecipitation. These cell lines, designated MNCP-2 and 20 (for Mengo Non-Capsid Polypeptide) were shown to produce immunoglobulins of the IgM and IgG_{2a} class, respectively.

Mengo viral polypeptides, both structural and non-structural, are produced by the cleavage of large precursor polypeptides. Fortunately however, unlike the capsid polypeptides, the non-structural polypeptides and their precursors are present in infected cells in monomeric form. This being the case, one would expect that a monoclonal antibody directed against a determinant in a particular non-capsid polypeptide would, by immunoprecipitation from

infected cell lysates, reveal precursor-product relationships. This expectation was borne out by an experiment the results of which are illustrated in Figure 6, a fluorogram of [^{35}S]-labeled polypeptides immunoprecipitated from an extract of infected cells by the monoclonal antibody produced by hybridoma line MNCP-20, and resolved by SDS-PAGE. As shown in lane 1, in the absence of 0.1% SDS in the immunoprecipitation buffers, monoclonal antibody MNCP-20 was found to immunoprecipitate polypeptide E, its precursors C and D, and small amounts of several other polypeptides including capsid polypeptides ϵ, α, β and γ which were incompletely removed from the cell extract by pre-adsorbtion with monoclonal antibody MCP-4. The capsid polypeptides were also immunoprecipitated non-specifically by a monoclonal antibody against polyoma virus VP1 (PyCp-3; Lane 2). The other, unidentified polypeptides immunoprecipitated by monoclonal antibody MNCP-20 are probably associated with the replication complex. Under more stringent conditions, i.e. in the presence of 0.1% SDS, non-specific immunoprecipitation was abolished, and only polypeptide E, with traces of its precursors C and D, was immunoprecipitated by monoclonal antibody MNCP-20 (lane 4). In this experiment, monoclonal antibody PyCP-3 appeared to immunoprecipitate a small amount of polypeptide E from the extract (lane 5). However, this was most likely due to contamination from the adjacent sample well, particularly in view of the failure of this monoclonal antibody to immunoprecipitate any polypeptide E

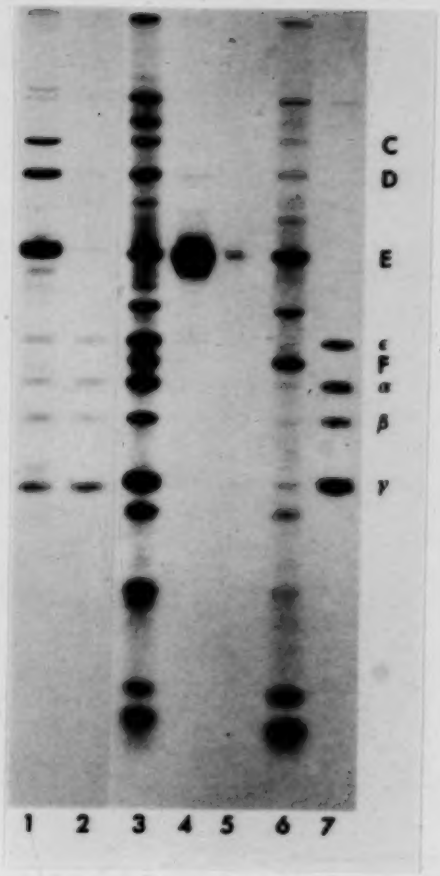

Figure 6. Fluorogram of [^{35}S]-labeled polypeptides present in and immunoprecipitated from an extract (S_{10}) of Mengo virus-infected L cells, and resolved by electrophoresis in an SDS-10% polyacrylamide slab gel. Lane 3: S_{10} extract of Mengo-infected cells. Lane 6: S_{10} extract after pre-adsorption with monoclonal antibody MCP-4. Lane 7: polypeptides immunoprecipitated from S_{10} extracts by monoclonal antibody MCP-4. Lanes 1 and 2: polypeptides immunoprecipitated in the absence of SDS from pre-adsorbed S_{10} extract by monoclonal antibodies MNCP-20 and PyCP-3 respectively. Lanes 4 and 5: polypeptides immunoprecipitated in the presence of 0.1% SDS from pre-adsorbed S_{10} extract by monoclonal antibodies MNCP-20 and PyCP-3 respectively.

under less stringent conditions (see lane 2).

Monoclonal antibody MNCP-2 (an IgM) was also found to be specific for polypeptide E by the technique of renaturing Western blotting as described in Chapter III (data not shown).

Purification of Polypeptide E by Immunoaffinity Chromatography

Highly purified polypeptide E was isolated from an $S_{4,3}$ extract of infected cells by an essentially one-step procedure in which the extract was passed sequentially through two columns prepared from Affi-Gel 10 to which anti-capsid monoclonal antibody MCP-4 (column 1) and monoclonal antibody MNCP-20 (column 2) were covalently linked, followed by elution of the pure protein from the latter. The results obtained from such a purification from an [^{35}S]methionine-labeled extract are illustrated in Figure 7. As may be seen, polypeptides were bound to column #1 (lane 5), and the extract, after passage through both columns, was found to be free of capsid polypeptides and polypeptide E, but to contain all the other non-structural viral polypeptides (lane 2). The only radiolabeled polypeptide eluted from column #2 by elution buffer was polypeptide E (lane 3). When the detergent NP-40 was eliminated from the elution buffer, recovery of polypeptide E was reduced by approximately 50% (data not shown).

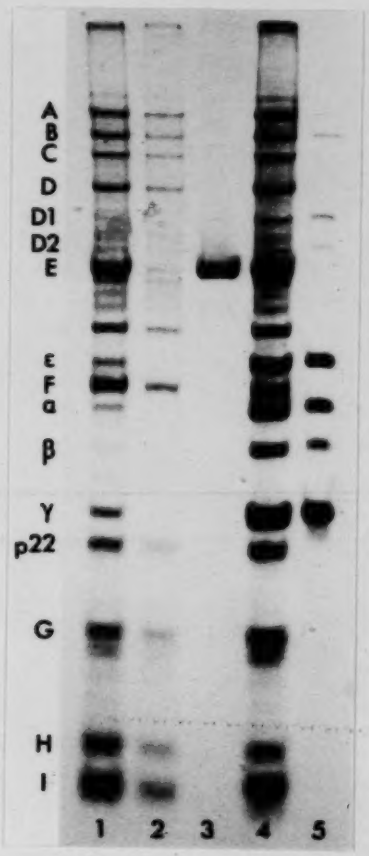

Figure 7. Fluorogram of [^{35}S]-labeled polypeptides present in an extract (S_{43}) of Mengo-infected L cells, and resolved by electrophoresis in an SDS-10% polyacrylamide slab gel. Lane 1: S_{10} extract of Mengo-infected cells. Lane 2: flowthrough polypeptides from combined anti-capsid and anti-E columns. Lane 3: polypeptides eluted from anti-E column. Lane 4: SDS extract of Mengo-infected cells. Lane 5: polypeptides eluted from anti-capsid column.

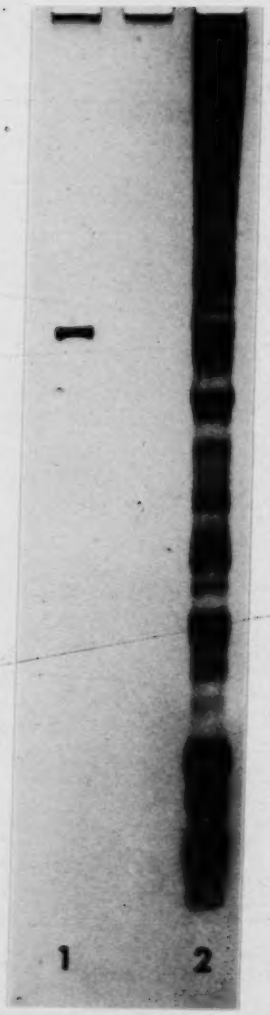

Figure 8. Electrophoresis of purified polypeptide E (lane 1) and of a sample of an SDS lysate of Mengo virus-infected cells (lane 2) in an SDS-10% polyacrylamide slab gel. Protein bands were visualized by silver staining.

The purity of polypeptide E isolated from a radiolabeled cell extract as outlined above was assessed further by silver staining (Bio-Rad silver stain kit) an SDS-polyacrylamide slab gel of the recovered protein to visualize contamination by unlabeled cellular proteins. As is clear from Figure 8, no such contaminants were detected.

Isolations of polypeptide E from extracts of cells infected with ts RNA$^-$ mutants of Mengo virus and with *wt* Mengo virus were equally successful. Typical densitometer scans of Coomassie Brilliant Blue R250 - stained electropherograms of purified replicase specified by the *wt* and one of the ts mutant viruses are shown in Figure 9. All polymerase preparations employed in the studies described here were shown by this technique to be > 95% pure.

The molecular weights of the immunoaffinity-purified polypeptides E specified by the *wt* and the RNA$^-$ ts mutants were estimated from their mobilities in SDS-10% polyacrylamide gels relative to those of standard marker proteins. The results are shown in Figure 10, from which it is clear that there are no significant differences among the *wt* and mutant replicases with respect to electrophoretic mobility. From these analyses, all were estimated to have an apparent molecular weight of 56,000.

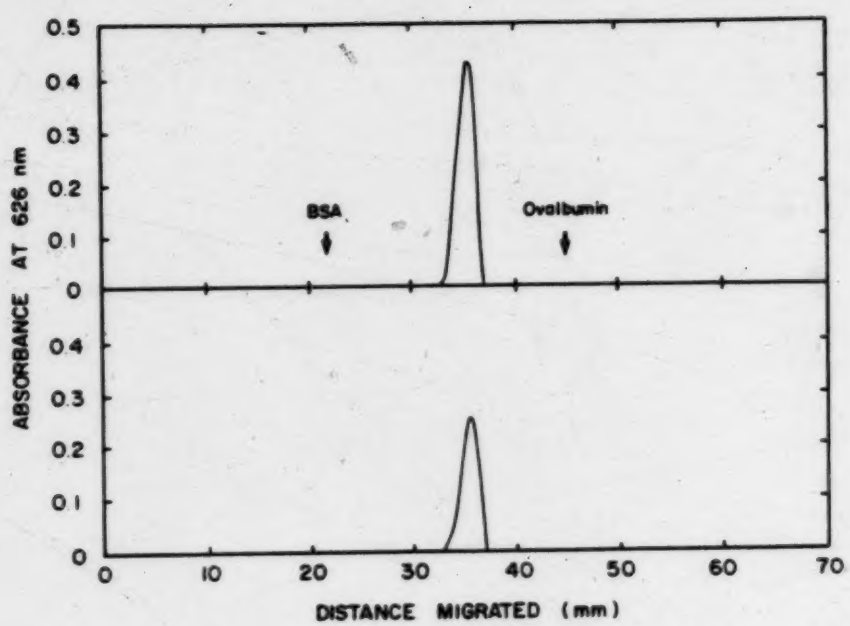

Figure 9. Densitometer scans of electropherograms (stained with Coomassie Brilliant Blue R-250) of purified polypeptide E specified by wt Mengo virus (upper panel; 2.5 μg) and by RNA⁻ mutant ts 506 (lower panel; 1.4 μg). The arrows in the upper panel indicate the relative mobilities of the proteins used to construct a standard curve used to quantitate polypeptide E in affinity column fractions.

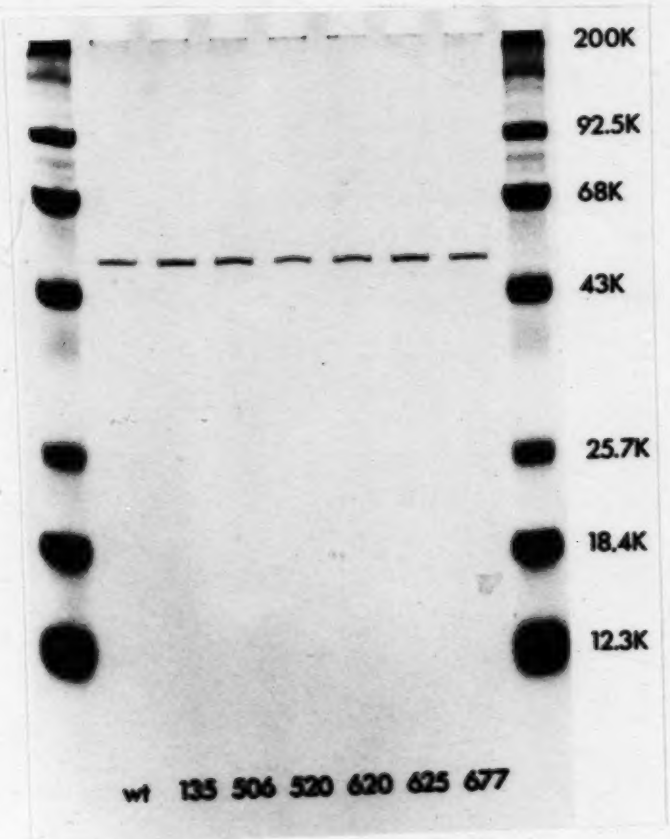

Figure 10. Comparison of the relative mobilities of wt and ts mutant-specified, immunoaffinity-purified polypeptides E (1 μg) resolved by SDS-PAGE and stained with Coomassie Brilliant Blue R-250. Flanking lanes contained BRL prestained high molecular weight standards.

Characterization of the Polymerase

A. Poly(U) polymerase Assay

Initial attempts to measure the biological activity of purified polypeptide E (specified by *wt* Mengo virus) utilized the assay system described by Baron and Baltimore (1982a), with an oligo(U) primer and RNA isolated from purified Mengo virions as the template. It was found that, in this system, polypeptide E is capable of catalyzing the incorporation of ^{3}H-CTP into TCA-insoluble products in the presence of unlabeled ATP, GTP and UTP. While this observation suggested that polypeptide E is capable of utilizing Mengo RNA to synthesize a complementary (negative) strand of RNA, the products of the reaction were not characterized. Since the main thrust of the investigations was to compare the activities of polypeptide E specified by the *wt* and ts RNA$^-$ mutants of Mengo virus, it was decided to use the somewhat simpler poly(U) polymerase assay. The results presented in this chapter were obtained using that system.

Preliminary studies of the *wt* immunoaffinity-purified polypeptide E revealed that the poly(U) polymerase activity of the molecule, as monitored by the incorporation of [^{3}H]UMP into TCA-insoluble material, is strongly dependent on the concentration of magnesium ions in the assay mixture. This finding is illustrated by the data shown in Figure 11, from which it was determined that the optimal [Mg^{++}] is 2mM. This concentration was used in all subsequent studies. The

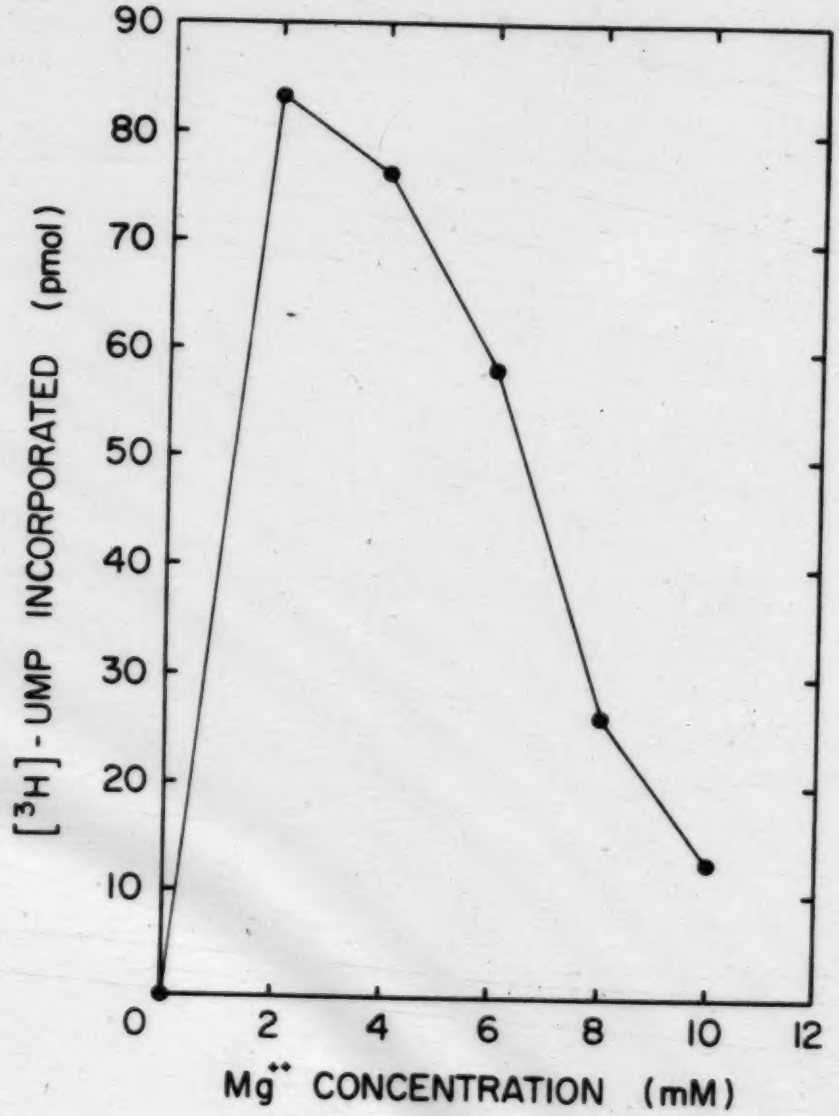

Figure 11. Effect of magnesium ion concentration on the poly(U) polymerase activity associated with the wt immunoaffinity-purified polypeptide E. The conditions employed were as described in Materials and Methods except that magnesium concentrations of 0, 2, 4, 6, 8 and 10mM were used. Incubation was for 30 min at 33°.

TABLE 5

Template, Primer and Cation Requirements for Poly(U)Polymerase Activity of Purified Polypeptide E

Modification of Assay System	Activity[b]
None (complete system)[a]	64.5
Complete system minus poly(A)	0.5
Complete system minus oligo(U)	0.5
Complete system minus Mg^{++}, plus 1mM $MnCl_2$	4.3
Complete system plus 0.2mM $ZnSO_4$	1.1

[a] Details of the composition of the 'complete' assay system are provided in the Materials and Methods section.

[b] pmoles [^{3}H]UMP incorporated into TCA-insoluble products. Incubation was for 30 min at 33°. Each mixture contained 0.4 µg purified polypeptide E (polymerase).

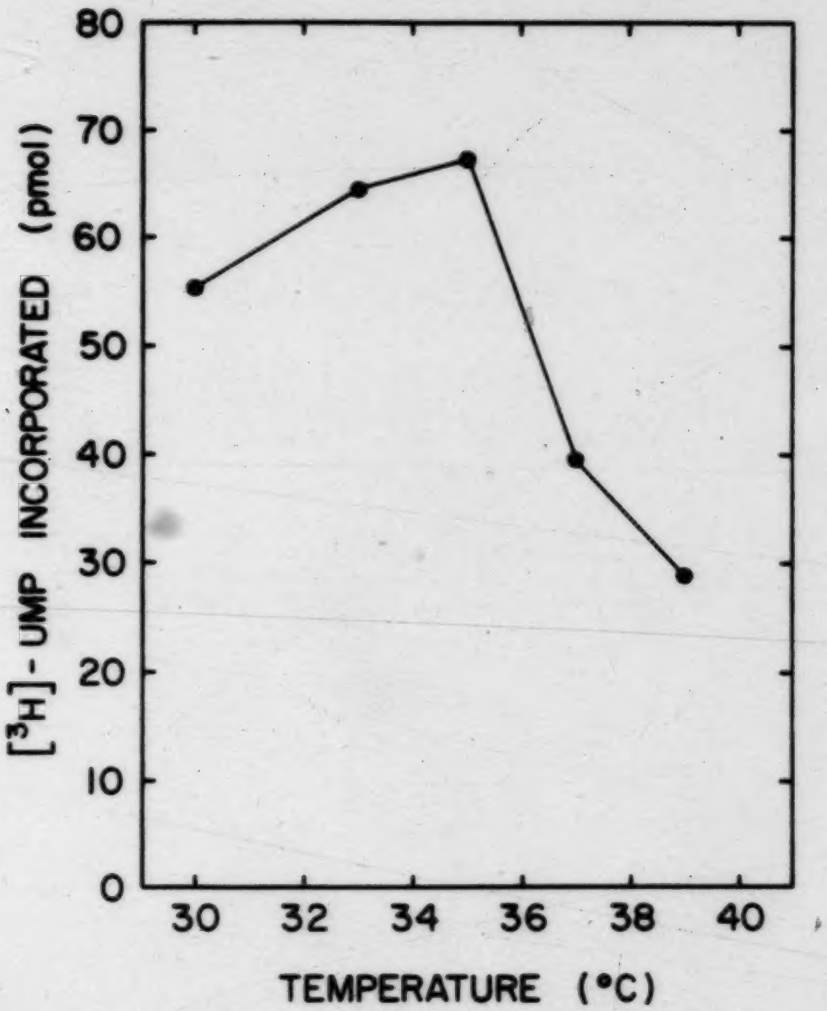

Figure 12. Poly(U) polymerase activity of the immunoaffinity-purified wt polypeptide E as a function of temperature. The assay was performed as described in Materials and Methods. Incubation was for 30 min at 30°, 33°, 35°, 37° and 39°.

template, primer and cation requirements for the polymerase activity of polypeptide E are listed in Table 5. The poly(U) polymerase activity was found to be dependent on both a poly(A) template and an oligo(U) primer, and it was shown that magnesium ions cannot be replaced by manganese ions as was reported to be the case for the poliovirus polymerase (Baron and Baltimore, 1982a). Also in contrast to earlier observations made with the poliovirus polymerase was the finding that zinc ions inhibit the activity of the Mengo polymerase. Baron and Baltimore (1982b) reported that this divalent ion stimulates the activity of partially-purified poliovirus polymerase. Actinomycin D (10μg/ml) has no affect on the polymerase activity (data not shown).

The temperature-activity profile obtained with the *wt* poly(U) polymerase is illustrated in Figure 12. Maximal incorporation of [^{3}H] UMP into TCA-insoluble form was found to occur at 35°, a temperature somewhat higher than that (30°) reported to be optimal for the poliovirus polymerase (Flanegan and Van Dyke, 1979).

The effect of monoclonal antibody MNCP-20 on the poly(U) polymerase activity of purified, *wt* polypeptide E was examined by incubating the latter with F(ab) fragments derived from antibody MNCP-20 before assaying the mixture for enzyme activity in the usual way. F(ab) fragments were used rather than intact IgG molecules to avoid dimerization of polypeptide E molecules due to cross-linking by antibody

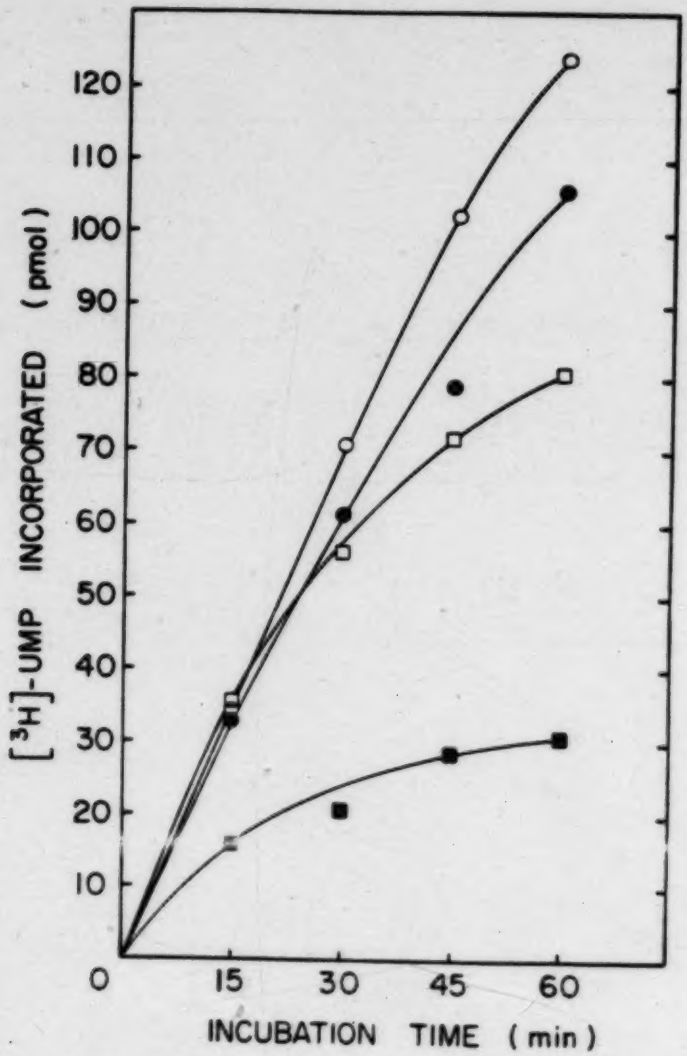

Figure 13. Effect of F(ab) fragments derived from monoclonal antibody MNCP-20 on the poly(U) polymerase activity of purified polypeptide E. The mixture of F(ab) and polypeptide E (1 molecule F(ab):1 molecule polypeptide E) was incubated for 2 hr at 4° before being assayed at 33° (circles) and 39° (squares). Open symbols: activities of F(ab) + polypeptide E mixture, closed symbols: activities of polypeptide E alone.

molecules. Incubation was for 2 hr at 4° at a ratio of 1 molecule of F(ab):1 molecule of polypeptide E. The rather surprising results are illustrated in Figure 13. Rather than inhibiting the enzyme as might have been expected, pre-incubation with F(ab) fragments was found to enhance the activity of the polymerase at both 33° and, to an even greater extent, 39°. The most reasonable explanation of this observation is that by interacting with polypeptide E, F(ab) molecules stabilize the enzyme by preventing conformational changes - and consequent loss of activity - that occur in their absence. Evidence that the effect was specific and not due simply to the presence of additional protein in the assay mixture was obtained by carrying out the same experiment with F(ab) fragments derived from a monoclonal antibody against capsid polypeptide β (MCP-6). With this preparation polymerase activities at both 33° and 39° were identical to those observed in the absence of added F(ab) fragments (data not shown).

B. Comparison of *wt* and ts Mengo Poly(U) Polymerases

The 6 ts Mengo isolates employed in these studies had been shown earlier by Downer *et al.* (1976) to be defective in viral RNA synthesis at the non-permissive temperature (39°). One possible explanation for the RNA$^-$ phenotype is that in each case the virus-specific polymerase synthesized in cells infected with the mutant virus is significantly more thermolabile than is the polymerase specified by the *wt* virus. This possibility was explored by measuring the

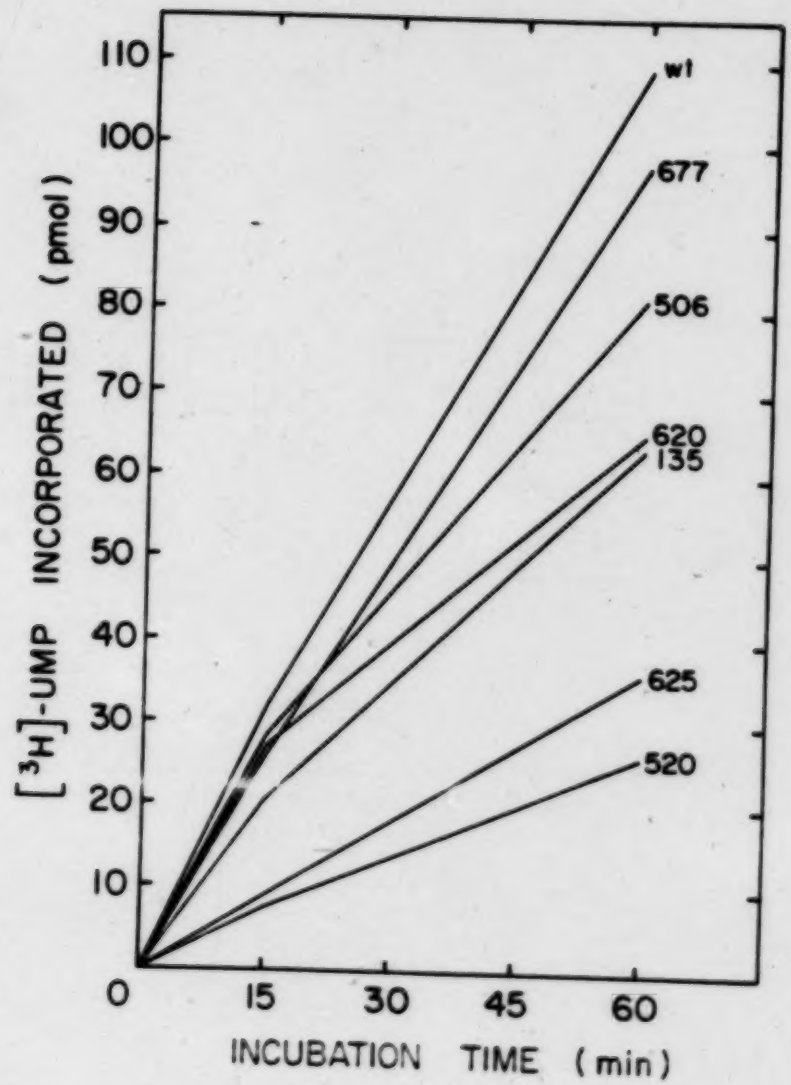

Figure 14. Poly(U) polymerase activity of polypeptide E specified by wt and by ts RNA⁻ mutants of Mengo virus. Assays were carried out at 33° as described in Materials and Methods. Each reaction mixture contained 4 μg of polypeptide E.

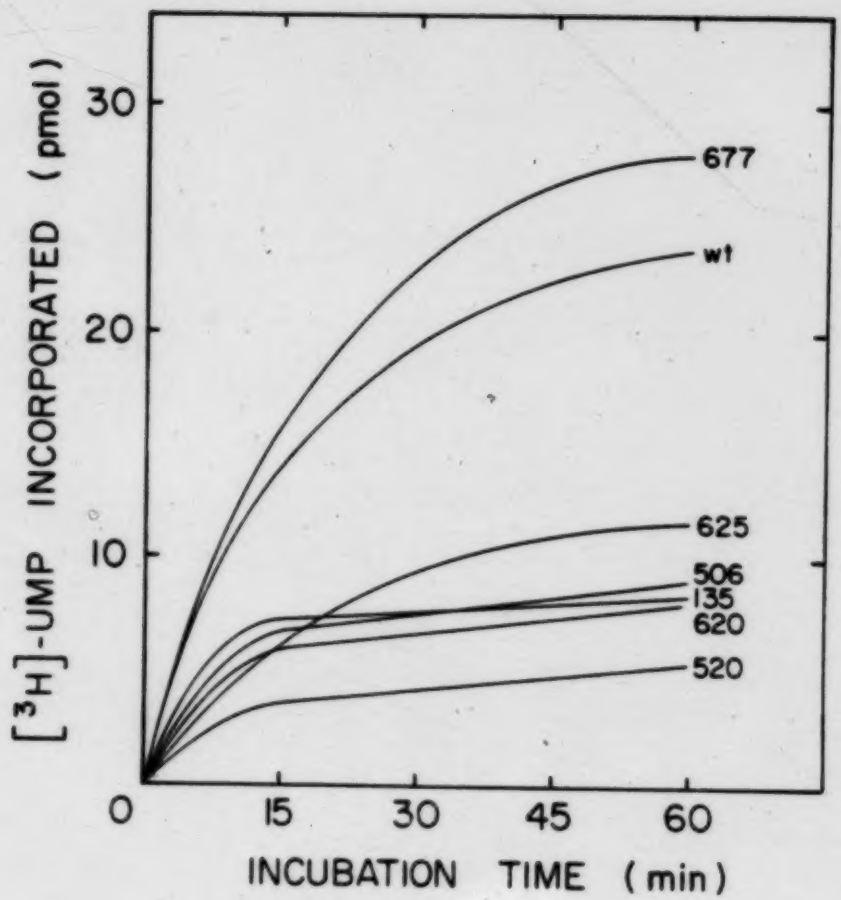

Figure 15. Poly(U) polymerase activity of polypeptide E specified by wt and by ts RNA⁻ mutants of Mengo virus. Assays were carried out at 39° as described in Materials and Methods. Each reaction mixture contained 4 μg of polypeptide E.

polymerase activities at 33° and 39° of purified polypeptides E specified by the *wt* virus and by the 6 ts RNA⁻ mutants. These temperatures are defined as the permissive and non-permissive respectively for the ts mutants (Downer *et al.*, 1976). It should be noted that the assay used measures the ability of the enzyme to synthesize poly(U) on a poly(A) template from an oligo(U) primer, - ie. it measures the elongation function of the enzyme only. In most cases, the incorporation of [^{3}H]UMP into TCA-insoluble product was found to be fairly linear with time for a period of 1 hr at 33°, as illustrated in Figure 14. It was somewhat surprising, however, to find such marked differences in activity among the polymerase preparations at this (at the permissive) temperature. At 39°, all polymerase preparations were found to be much less active than at 33°, and in all cases the incorporation of ^{3}H-UMP into TCA-precipitable products expressed as a function of time was found to deviate significantly from linearity (Figure 15).

As an indication of the relative thermostabilities of the *wt* and ts mutant poly(U) polymerases, the incorporation of ^{3}H-UMP (in pmoles) into TCA-insoluble products after 1 hr of incubation is given in Table 6. The data indicate that the poly(U) polymerase specified by mutant ts 677 is the most stable, that the enzyme specified by the *wt* virus is of intermediate stability and that those specified by mutants ts 135, ts 506, ts 520, ts 620 and ts 625 are the most thermolabile.

TABLE 6

Thermostability of wt and ts Mengo Poly(U) polymerases

Virus-specific Polymerase	Activity of enzyme at 39°[a]
wt	23.7
ts 135	8.3
ts 506	9.1
ts 520	5.4
ts 620	7.9
ts 625	11.0
ts 677	27.9

[a] Activities, expressed as pmoles [^{3}H]UMP incorporated into TCA-insoluble products after 1 hr of incubation, were taken from the experiment illustrated in Figure 15.

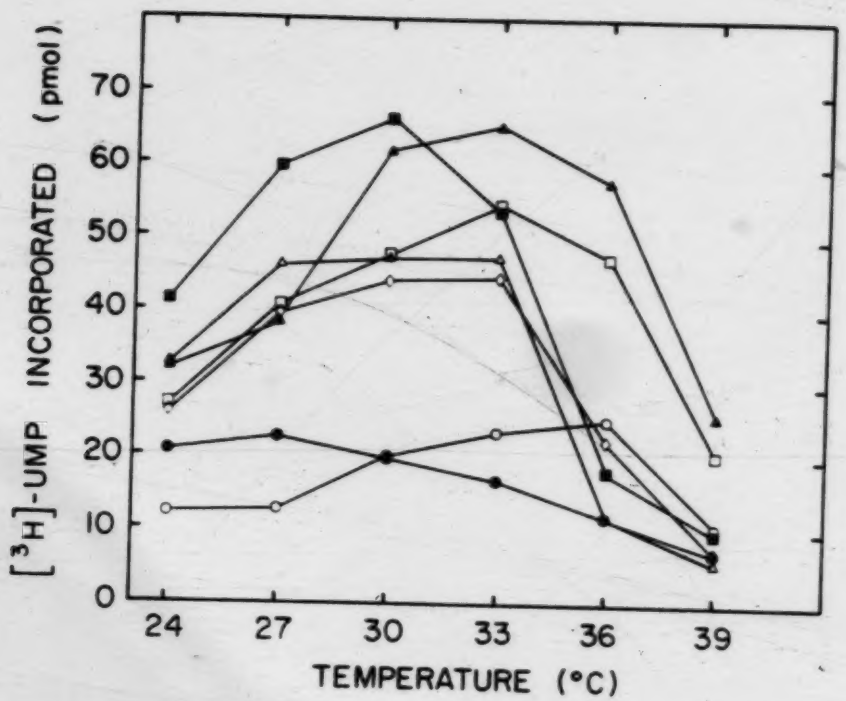

Figure 16. Effect of incubation temperature on the poly(U) polymerase activity of purified polypeptides E specified by wt and by ts RNA$^-$ mutants of mengo virus. Assay mixtures were as described in Materials and Methods. Incubations were for 30 min at the indicated temperatures. (□) wt, (◇) ts 135, (■) ts 506, (●) ts 520, (△) ts 620, (○) ts 625 and (▲) ts 677.

The observed differences among the *wt* and ts mutant polymerases with respect to thermostability prompted an investigation of the optimum temperature for each. The results of this study are shown in Figure 16. The most stable of the polymerases, that specified by mutant ts 625 was found to be maximally active at 36°, whereas the *wt* and ts 677 polymerases exhibited peak activity between 33° and 36°. The less stable ts 135 and ts 620 polymerases showed a plateau of activity between 30° and 33° with a precipitous drop in activity at higher temperatures, and the ts 506 polymerase was found to be maximally active at 30°. The lowest temperature optimum (27°) was exhibited by the ts 520 polymerase, although its activity decreases very slowly with increasing temperature.

C. Circular Dichroism and Fluorescence Studies

Perhaps the most obvious explanation of the observed thermal inactivation of the poly(A)-dependent poly(U) polymerase activity of polypeptide E, specified by the *wt* virus as well as by the ts mutants, is that the loss of activity reflects temperature-induced conformational changes in this molecule. Since measurements of the circular dichroism and the tryptophan fluorescence of proteins provide sensitive probes for detecting conformational changes therein, a careful investigation of the various purified polypeptides E by these techniques was undertaken.

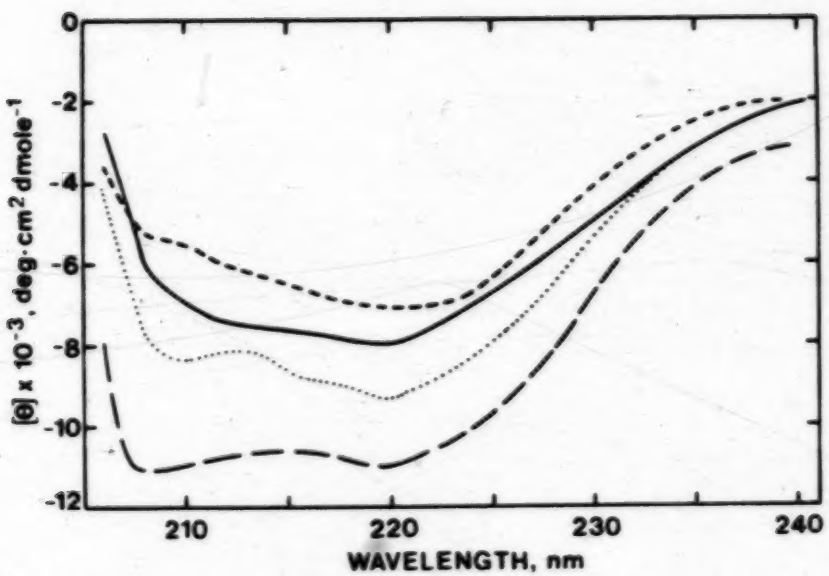

Figure 17. Far ultraviolet circular dichroism of the immunoaffinity-purified wt polypeptide E (the poly(U) polymerase) in 50mM sodium phosphate, pH 7.0 containing 0.02% octaethylene glycol, measured as described in Materials and Methods (——) 24.8°, (·····) 29.4°, (——) 34.8° and (-----) 39.5°.

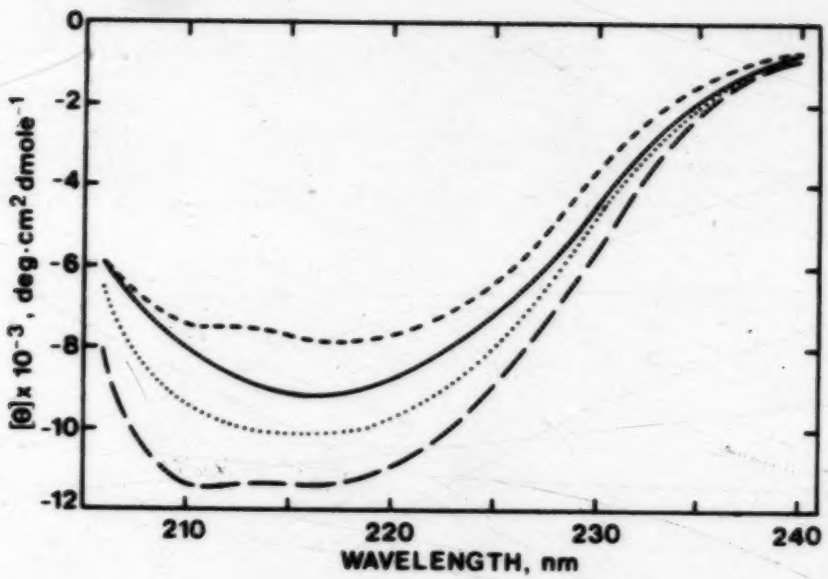

Figure 18. Far ultraviolet circular dichroism of the immunoaffinity-purified ts 677 polypeptide E in 50mM sodium phosphate, pH 7.0 containg 0.02% octaethylene glycol, measured as described in Materials and Methods. (— —) 25.2°, (·····) 29.5°, (——) 34.0° and (----) 39.3°.

Illustrative circular dichroic spectra, measured in the far ultraviolet at temperatures between 25° and 40°, are shown in Figures 17 and 18 for the *wt* and ts 677 polypeptides E, respectively. Each set of curves shows a progressive decrease in the negative ellipticity of the corresponding protein with increasing temperature. For example, the negative ellipticity at 222nm, which is due primarily to the α-helical content of the molecule, was found to decrease with increasing temperature. It is clear that both polypeptides, as well as the other 5 ts polypeptides whose spectra are not shown here, undergo significant, temperature-induced changes in secondary structure between 25° and 40°.

The molar ellipticity of each of the polypeptides E at 25° was determined for the corresponding circular dichroic spectrum, and from that parameter, the secondary structure (expressed as the per cent of α-helix, β-structure and random coil conformations) of each polypeptide E at 25° was calculated as described in Appendix I. The results are summarized in Table 7. The average of the values obtained with all the polypeptides indicate that the secondary structure of the poly(U) polymerase contains 34% α-helix, 20% β-structure, and 46% random coil. It has been estimated that the experimental error involved in measuring circular dichroic spectra is of the order of 2.5-4%. On this basis, it may be concluded that, with the possible exception of ts 677 polymerase (29.1% β-structure and 38% random coil), the

TABLE 7

Secondary Structures of the wt and ts Mengo Poly(U) Polymerases[a]

Polymerase isolated from	α-helix	β-structure	Random coil
wt virus	34.9	19.7	45.4
ts 135	32.0	22.0	46.0
ts 506	34.0	24.0	42.0
ts 520	35.3	18.9	45.8
ts 620	33.8	18.8	47.4
ts 625	34.3	23.9	41.8
ts 677	32.9	29.1	38.0

[a] Calculated from the circular dichroic molar ellipticities using the method of Chen et al. (1974) as described in Appendix I. Secondary structures are expressed in terms of the % of each molecule in the α-helix, β-structure and random coil conformations.

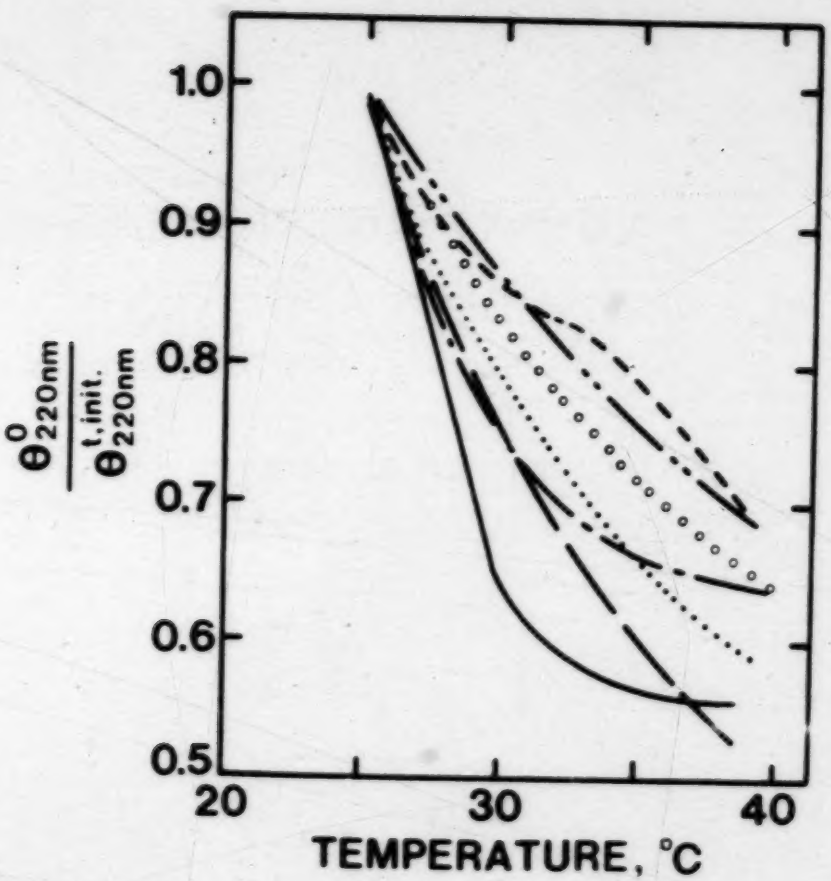

Figure 19. Relative ellipticities of the wt and ts Mengo poly(U) polymerases, calculated from circular dichroic measurements as described in Materials and Methods. (ooooo) wt, (——) ts 135, (———) ts 506, (·····) ts 520, (----) ts 620, (-———-—) ts 625, and (--———--——) ts 677.

secondary structures of the ts mutant polymerases at 25° do not differ significantly from that of the *wt* polymerase.

However, calculations of relative ellipticities as described in Materials and Methods did reveal differences among the polypeptides E with respect to conformational stability. This is illustrated in Figure 19, in which the relative ellipticity of each polypeptide is plotted as a function of temperature. The steeper the slope of the curve, the more rapidly is secondary structure melted out as the temperature is increased. As may be seen, ts 620 and ts 677 polypeptides, with relative ellipticities higher than that of the *wt* polypeptide, are the most stable members of the group, while the ts 135, ts 506 and ts 520 polypeptides are less stable than the *wt* polypeptide. The ts 625 polypeptide is also less stable than the *wt* polypeptide, although at the upper end of the temperature range its stability approaches that observed with the *wt* polypeptide.

The circular dichroism of the complex between the *wt* polypeptide E and the F(ab) fragment derived from monoclonal antibody MNCP-20 was also examined. The conformation (secondary structure) of the F(ab) fragment alone was found to be stable under the experimental conditions employed. This is illustrated by Figure 20, which shows that the CD spectra of the protein measured at 25°, at 40°, and after heating the sample to 40° and then re-cooling it to 25°, do not differ significantly. Circular dichroic spectra of the

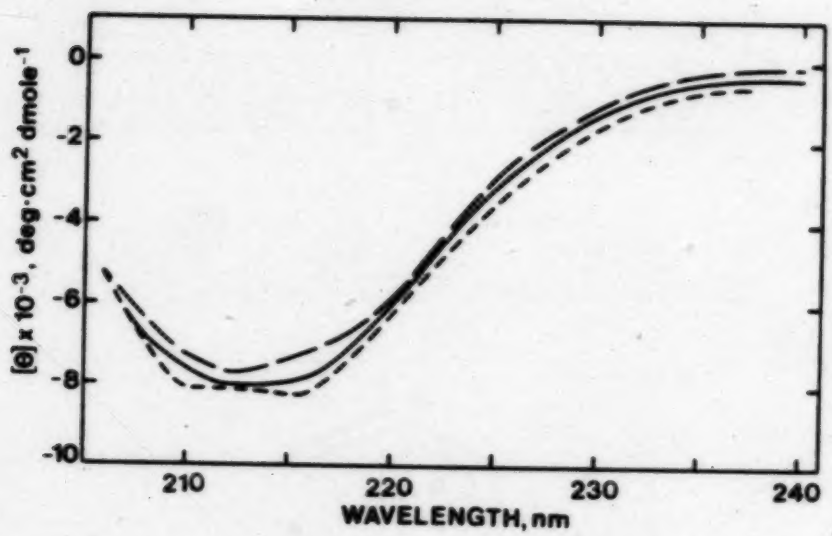

Figure 20. Circular dichroism of the isolated F(ab) fragment of monoclonal antibody MNCP-20 in 50mM sodium phosphate, pH 7.0 containing 0.02% octaethylene glycol, measured as described in Materials and Methods. (— —) 25.0°, (——) 40.8° and re-cooled to (----) 25.0°.

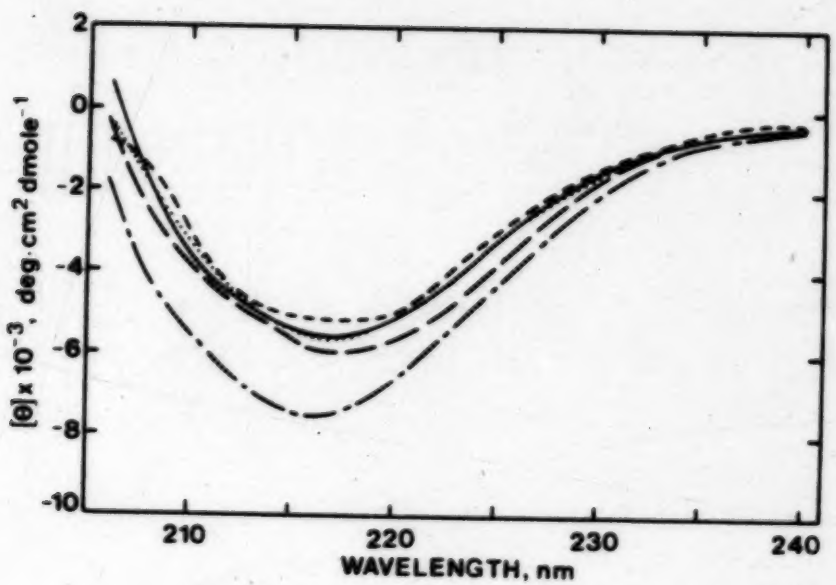

Figure 21. Circular dichroism of the complex between the wt polypeptide E and the F(ab) fragment derived from monoclonal antibody MNCP-20, in 50mM sodium phosphate, pH 7.0 containing 0.02% octaethylene glycol. The purified wt polypeptide E was incubated for 2 hr at 4° with the anti-E F(ab) fragment (1:1 ratio of molecule of F(ab) to molecules of polypeptide E) before CD measurements were made as described in Materials and Methods (— —) 24.5°, (——) 29.4°, (·····) 34.0°, (----) 39.0° and re-cooled to (-·—·-) 25.0°.

wt polypeptide E -F(ab) complex, measured at several temperatures between 24.5° and 39°, and again after cooling the sample to 25°, are presented in Figure 21. It is clear from the very small spectral shifts that were observed as the temperature was increased, that the conformation of the complex is much more stable than is that of the viral polypeptide alone (see Figure 17). Upon re-cooling the sample to 25°, the complex was found to assume a more stable conformational state than that in which it existed initially, as evidenced by the increased negative ellipticity of 1500 $deg.cm^2dmole^{-1}$ at 217nm. The response of the complex to re-cooling is in sharp contrast to that exhibited by the polypeptides (*wt* and ts) themselves, all of which were found to undergo marked, temperature-induced conformational changes that were not reversed by cooling. The increased conformational stability of the polypeptide E resulting from the formation of a complex with the F(ab) fragment is consistent with the observation that the poly(U) polymerase activity of E is markedly enhanced (stabilized) particularly at 39°, in the presence of the antibody fragment.

Temperature-induced changes in the conformation of a protein can also be detected by monitoring its intrinsic fluorescence, which is due to the presence of fluorescent chromophores (the aromatic side chains in tryptophan, tyrosine, and phenylalanine) in the molecule, and which almost always decreases with increasing temperature. Measurements of the intrinsic fluorescence of the purified *wt*

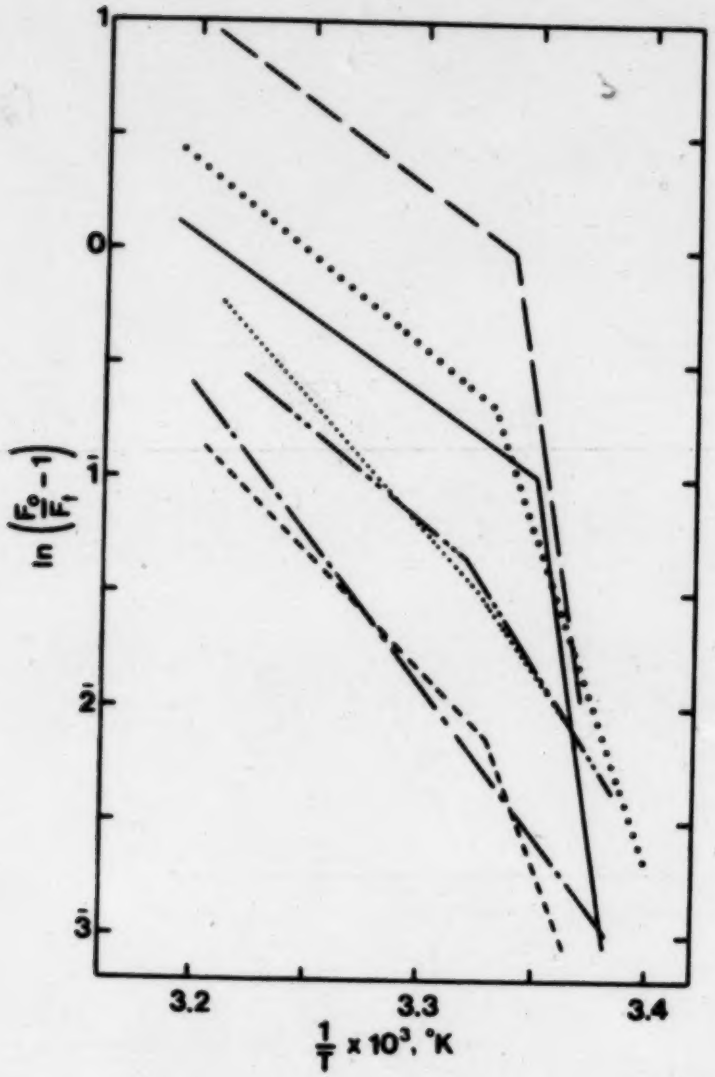

Figure 22. Arrhenius plots ($\ln (F_o/F_t - 1)$ vs reciprocal of absolute temperature) based on measurements of the intrinsic fluorescence of purified wt and ts Mengo poly(U) polymerases in 50mM sodium phosphate, pH 7.0, containing 0.02% octaethylene glycol. Fluorescence was measured at 333nm following excitation at 280nm. F_t is the fluorescence at higher temperatures. Virus-specific wt (ooooo), ts 135 (——), ts 506 (——), ts 520 (·····), ts 620 (----), ts 625 (-—-—), and ts 677 (--———) polymerases.

TABLE 8

Relative Conformational Stabilities of the wt and ts Mengo Poly(U) Polymerases as Determined from Measurements of Intrinsic Fluorescence

Virus-specific polymerase	Break Point[a] (in °C)
wt	27.0
ts 135	26.0
ts 506	25.5
ts 520	28.0
ts 620	27.0
ts 625	-
ts 677	28.0

[a] Determined from the Arrhenius plots in Figure 22.

and ts poly(U) polymerases were carried out at temperatures between 25° and 40°, with fluorescence emission being measured at 333nm following excitation of the molecules at 280nm. As indicated in Materials and Methods, measuring the fluorescence at this wavelength focuses on changes in the conformation of those parts of the protein molecule adjacent to tryptophan residues, since the emission maximum of this amino acid is at or very close to 333nm. The results of this study are presented in the form of Arrhenius plots in Figure 22. The break points (points of intersection of the two linear segments of the plots), which correspond to the transition temperatures between two conformational states of the polymerases, were estimated from the Arrhenius plots shown in Figure 22, and are listed in Table 8.

These estimates indicate that the poly(U) polymerases specified by mutants ts 520 and ts 677 are more stable, and that those specified by mutants ts 135 and ts 506 are less stable, than the *wt* polymerase. The ts 620 and *wt* polymerases appear to have the same conformational stability. The Arrhenius plot obtained with the polymerase specified by mutant ts 625 does not have a break point, which suggests that it is the most stable of the polymerases examined, at least with respect to the conformation of the molecule close to tryptophan residues, and over the temperature range employed. The temperatures corresponding to the break points in these Arrhenius plots are somewhat lower than might have been predicted from the temperature-activity profiles shown

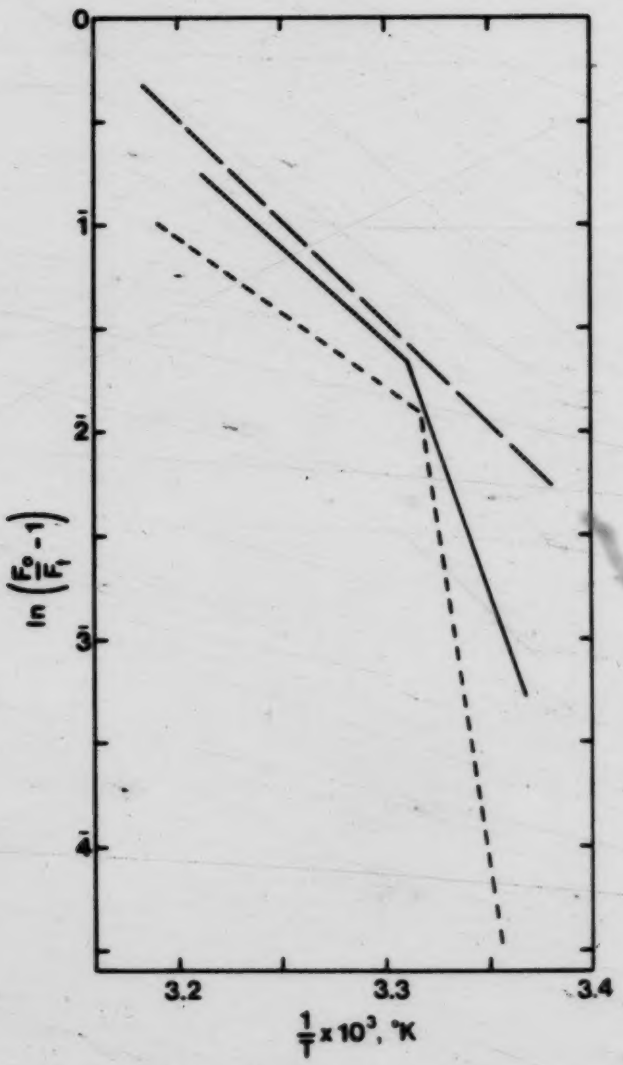

Figure 23. Arrhenius plots based on measurements of the intrinsic fluorescence of the wt Mengo poly(U) polymerase (——), the F(ab) fragment of monoclonal antibody MNCP-20 (-----), and the F(ab)-polymerase complex (——). See legend to Figure 22 for further details.

in Figure 16. This, however, may be an artefact of the experimental procedure used, since the temperature-induced changes in the conformation of the poly(U) polymerases (as reflected by decreases in intrinsic fluorescence) are not fully reversible on re-cooling (data not shown), and this may have resulted in a premature shift in the equilibrium state.

The stabilization of the conformation of the *wt* polymerase by the formation of a complex with the F(ab) fragment of monoclonal antibody MNCP-20 can also be demonstrated by fluorescence measurements, as illustrated in Figure 23. Arrhenius plots constructed from fluorescence measurements of the separate components exhibit well-defined break points, estimated, in the experiment shown here, to be at 28.0° and 29.0° for the F(ab) fragment and polymerase respectively. The Arrhenius plot based on fluorescence measurements of the F(ab)-polymerase complex has no break point, indicating that it is stable over the temperature range employed.

D. Amino Acid Composition and N-terminal Sequence Analysis

The amino acid compositions of the purified *wt* polypeptide E and the polypeptides E specified by four of the ts RNA$^-$ Mengo mutants are listed in Table 9. It was not possible to determine the values for cysteine and tryptophan because of the limited quantities of the purified polypeptides that were available. No significant differences in

TABLE 9

Amino Acid Composition[a] of the wt and ts Mengo Poly(U) Polymerases (Polypeptides E)

	wt	ts 135	ts 506	ts 520	ts 625	EMC virus[b]
Aspartate/ Asparagine	9.9	9.8	9.6	9.9	9.7	8.9
Threonine	6.7	6.6	6.6	6.8	6.5	8.0
Serine	5.8	5.8	6.0	6.3	5.7	5.8
Glutamate/ Glutamine	10.4	10.8	10.5	10.6	10.6	9.7
Proline	5.1	4.9	4.8	5.1	5.2	6.1
Glycine	6.5	6.7	6.7	6.6	6.6	5.8
Alanine	7.8	7.9	7.8	7.7	7.8	7.6
Valine	7.2	7.4	7.5	7.3	7.3	7.6
Methionine	2.7	2.1	2.2	1.7	2.7	2.6
Isoleucine	4.1	4.3	4.3	4.2	4.3	3.0
Leucine	10.5	10.5	10.3	10.6	10.4	10.2
Tyrosine	3.7	3.5	3.6	3.5	3.5	3.7
Phenylalanine	5.5	5.4	5.4	5.3	5.4	6.5
Histidine.	1.7	1.9	2.1	1.8	2.0	1.5
Lysine	6.1	6.3	6.1	6.2	6.0	6.3
Arginine	6.5	6.2	6.3	6.4	6.3	6.7

[a] Determined as described in Materials and Methods and expressed in nmoles %.

[b] Determined from the sequence of EMC virus (Palmenberg et al., 1984) excluding cys and trp residues.

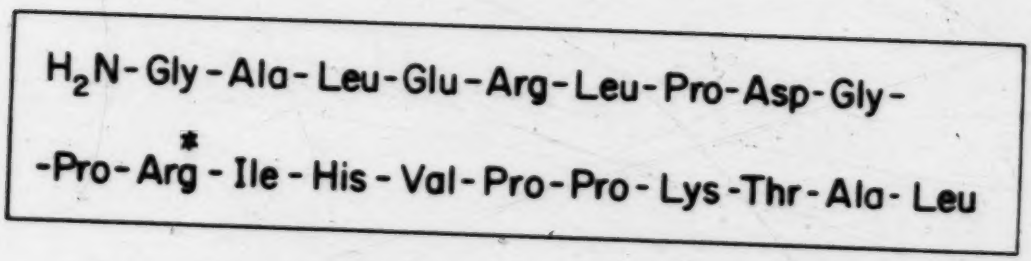

Figure 24. Amino terminal sequence of the purified wt and ts 520 polypeptides E. Sequence analyses were performed by automated Edman degradation, as described in Materials and Methods.

the amino acid compositions of the *wt* and ts Mengo polypeptides E were found, an observation consistent with the view that a very limited number of point mutants were introduced into the genomes of these ts mutants by nitrous acid, the agent that was used to mutagenize the stock virus from which they were isolated. The amino acid composition of the *wt* polypeptide E shown here is very similar to the composition calculated from the deduced sequence of the EMC virus polypeptide E (see Table 9). This might suggest that the apparent sequence homology seen with the capsid polypeptides of these two serologically-indistinguishable viruses might also extend into the polypeptide E region of the genome.

The N-terminal sequences of the *wt* and ts 520 polypeptides E, determined by automated Edman degradation, are shown in Figure 24. The 16 amino terminal residues of the *wt* and ts 520 polypeptides E were found to be identical except for some microheterogeneity at position 11 in the ts 520 polypeptide (Arg* in Figure 24) where a mixture of leucine and arginine (approximately 1:1) was found. The sequence was extended by an additional 4 residues (to position 20) with the ts 520 polypeptide. It is of interest to note that the amino terminal sequence shown here is identical to that of the corresponding polypeptide of EMC virus as deduced from the nucleotide sequence of the cDNA corresponding to the EMC viral genome (Palmenberg *et al.*, 1984), except that in the deduced primary structure of the EMC polymerase the proline at position 16 is replaced by an arginine residue. In this

regard, it should be pointed out that, for two reasons, there is considerable uncertainty with respect to the identification of proline at position 16: 1) arginine is detected rather inefficiently by chromatography of its phenylthiohydantoin derivative, and 2) proline is cleaved less efficiently from the polypeptide than are the other amino acids during the Edman degradation and as a result can be a significant contaminant of the following cycle.

Discussion

The isolation of a hybridoma cell line that produces a monoclonal antibody against Mengo virus-specified polypeptide E (poly(U) polymerase) has made it posssible to isolate this protein in homogeneous form by immunoaffinity chromatography. As was reported earlier for the partially purified poliovirus poly(U) polymerase (Flanegan and Baltimore, 1977), the isolated polypeptide E requires a template, a primer and magnesium ions for polymerase activity. Data obtained from measurements of the poly(U) polymerase activity of the purified *wt* polymerase at 33° and 39° show that it is thermolabile, and measurements of circular dichroism and tryptophan fluorescence demonstrate clearly that the decrease in the activity of the enzyme at higher temperatures coincides with a change in its conformation (secondary structure). The finding that both the enzyme activity and the secondary structure of the polymerase are stabilized (against heat-induced loss/change) by the

formation of a complex with the F(ab) fragment derived from the anti-polypeptide E IgG provides additional evidence that the heat inactivation of the polymerase (in the absence of the F(ab) fragment) is the direct result of a conformational change in the enzyme. The results also lead one to conclude that the antigenic determinant corresponding to the monoclonal antibody used in these studies is not located at or near the active center of the polymerase, and to speculate that the apparent *in vivo* stability of the enzyme is due to interaction with other virus-specific and/or host proteins that play a role in the replication of the viral genome.

One of the objectives of the investigations described here was to determine whether the RNA$^-$ phenotype exhibited by the 6 ts RNA$^-$ mutants used in the studies is due to temperature sensitive lesions in the mutant-specified polymerases. The fact that the purified *wt* poly(U) polymerase is itself thermolabile makes interpretation of the data more difficult, since at best, one can describe each ts polymerase as being somewhat more or somewhat less thermolabile than the *wt* enzyme. It is important to bear in mind also, that the RNA$^-$ phenotype of the mutants may be due to one of several factors including: 1) a temperature-sensitive lesion in the polymerase which, at the non-permissive temperature, results in the loss of either the elongation activity of the enzyme (the activity which was probably measured in the oligo(U)-poly(A) dependent assay), or its ability to interact with protein initiation

TABLE 10

Summary of the Properties of the Isolated Virus-encoded Poly(U) Polymerases

Virus-specific polymerase	Activity at 39°	Optimal Temperature	Conformational stability[a] by CD	by fluorescence
wt	23.7	33°-36°	--	--
ts 135	8.3	33°	less	less
ts 506	9.1	33°	less	less
ts 520	5.4	27°	less	more
ts 620	7.9	27°-33°	more	same
ts 625	11.0	36°	same	more
ts 677	27.9	33°-36°	more	more

[a] Relative to that of the wt polymerase.

factors (host or viral) while leaving its elongation function unaffected; 2) a temperature-sensitive lesion in some other viral-coded polypeptide that may be required for initiation *in vivo*; or 3) faulty processing (cleavage) of a precursor polypeptide (of either the polymerase or other essential viral polypeptide) at the non-permissive temperature. These considerations notwithstanding, it is possible to draw certain conclusions from the measurements of enzyme activity and of circular dichroism and tryptophan fluorescence described herein. To facilitate comparisons of the various polymerases, the essential features of the data obtained from these studies are summarized in Table 10.

It is clear from these data (obtained from both enzyme assays and physical measurements) that the poly(U) polymerase specified by mutant ts 677 is more stable than the *wt* polymerase, from which one can conclude that the RNA$^-$ phenotype of this mutant is not due to inactivation of the elongation function of its polymerase at the non-permissive temperature. In contrast, the data suggest that the RNA$^-$ phenotype exhibited by mutants ts 135 and ts 506 can be attributed directly to temperature-sensitive lesions in the polymerase which they encode. Both polymerase were shown, by both enzyme assay and spectral analyses, to be more thermolabile than the *wt* enzyme.

The data obtained with the other three ts mutants are more difficult to interpret. On the basis of the poly(U)

polymerase assay, ts 620 enzyme activity seems clearly to be more thermolabile than that of the *wt*. However, the correlation between the results of the enzyme assays and measurements of circular dichroism and tryptophan fluorescence is not as clear cut as is the case with the ts 135 and ts 506 polymerases. On the basis of fluorescence measurements, the conformational stabilities of the ts 620 and *wt* polymerases appear to be essentially the same. On the other hand (see Figure 19) circular dichroism measurements indicate that the relative ellipticity of the ts 620 polymerase is somewhat higher than that of the *wt* enzyme over the whole temperature range (25°-40°) used, which suggests that it has a more stable secondary structure than the *wt* enzyme. However, at temperatures above 33°, the upper limit of a relatively broad temperature range (27°-33°) over which the ts 620 polymerase is optimally active (see Figure 16), both the polymerase activity and the relative ellipticity of the ts 620 polymerase decrease more rapidly than do these parameters of the *wt* enzyme. Taken together, these observations make it seem likely that the RNA$^-$ phenotype of mutant ts 620 is also due to a temperature-sensitive lesion which affects the elongation function of its polymerase.

Even more difficult to interpret are the data obtained with mutants ts 520 and ts 625. Examination of the temperature-activity profiles shown in Figure 16 shows that the poly(U) polymerases specified by these two mutants - unlike those specified by the other four mutants - do not,

at any temperature, have specific activities even close to that exhibited by the *wt* enzyme at its optimal temperature. This suggests that, at all temperatures, both enzymes may have a decreased affinity for the template-primer and/or the substrate. The temperature-activity profiles obtained with these two polymerases are relatively flat, and the circular dichroism and fluorescence measurements indicate that over the temperature range employed both undergo less marked conformational changes than does the *wt* enzyme (and that the ts 625 polymerase is the most stable of all the polymerases examined). Nonetheless, the low optimal temperature (27°) for the poly(U) polymerase activity of ts 520 polypeptide E (see Figure 6) and the finding that with this polypeptide the rate of incorporation of ^{3}H-UMP into TCA=insoluble products at 39° decreased dramatically after 15 min incubation (see Figure 15), suggests that the RNA$^-$ phenotype of this mutant can also be explained onn the basis of a temperature-sensitive lesion in polypeptide E.

In the case of mutant ts 625, earlier studies (pulse-chase experiments) of the synthesis of viral polypeptide at the permissive and non-permissive temperatures suggest an alternative explanation. In cells infected with this mutant there appears to be faulty cleavage of polypeptide C and D (precursors of polypeptide E) and possible further cleavage of polypeptide E at the non-permissive temperature, all of which result in the appearance of three abnormal products of molecular weights 41, 25.5 and 16K

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(data not shown). This aberrant cleavage pattern, in conjunction with the low specific activity of the ts 625 polymerase may account for the RNA$^-$ phenotype of mutant ts 625.

A logical extension of the studies described here would be to investigate the replication of Mengo RNA in an *in vitro* system. Given the availability of completely homogeneous polypeptide E, it would be possible to attempt to reconstitute the complete system required for the replication of the viral genome, and to identify and define the roles of other virus-specific and/or host factors that may be involved in the process.

Chapter V

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Appendix I

Molar ellipticities were calculated from the observed ellipticities at 25° employing the following equation:

$$[\theta] = \frac{\theta_{obs} \times MRW}{10 \times 1 \times C}$$

where $[\theta]$ = molar ellipticity at a given wavelength, θ_{obs} = observed ellipticity in degrees, MRW = mean residue weight (taken as 115 g), 1 = path length in cm and c = concentration in g/ml.

The per cent α-helix, β-sheet and random coil were calculated using the molar elipticites at four different wavelengths (210, 215, 220 and 225nm) by the method of Chen et al. (1974). The values reported were average values. The equations employed were:*

Equation 1. Values of $[\theta]$ taken at 210 and 225nm

$(((\theta_{210} - 2200) \times 1276) + ((\theta_{225} + 264) \times 5990))\ 204373560 = \alpha$

$((\theta_{210} - 2200) - (24100 \times \alpha))\ 5990 = \beta$

Equation 2. Values of $[\theta]$ taken at 215 and 225nm

$(((\theta_{215} + 669) \times 1276) - ((\theta_{225} + 264) \times 10009))\ 323547520 = \alpha$

$((\theta_{215} + 669) - (26369 \times \alpha))\ 10009 = \beta$

Equation 3. Values of $[\theta]$ taken at 220 and 225nm

$(((\theta_{220} + 1800) \times 1276) + ((\theta_{225} + 264) \times 7860))\ 267595840 = \alpha$

$(\theta_{220} + 1800) - (31300 \times \alpha)\ 7860 = \beta$

The per cent random coil was determined by subtraction.

*The constants used are the reference values for pure α-helix, β-structure and random coil computed at each wavelength from several reference proteins.